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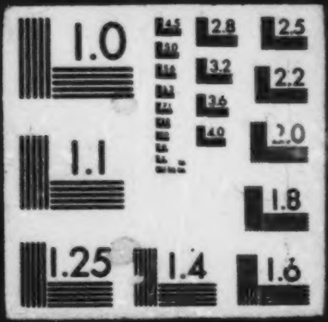
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UPTAKE AND UTILIZATION OF AMINO ACIDS DURING ANAEROBIC DIGESTION

Ernest M. Miholits, et al

Texas University
Austin, Texas

November 1965

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Technical Report
to
WATER SUPPLY AND POLLUTION CONTROL DIVISION
U. S. PUBLIC HEALTH SERVICE

UPTAKE AND UTILIZATION OF AMINO ACIDS DURING ANAEROBIC DIGESTION

SEAL OF THE UNIVERSITY OF TEXAS

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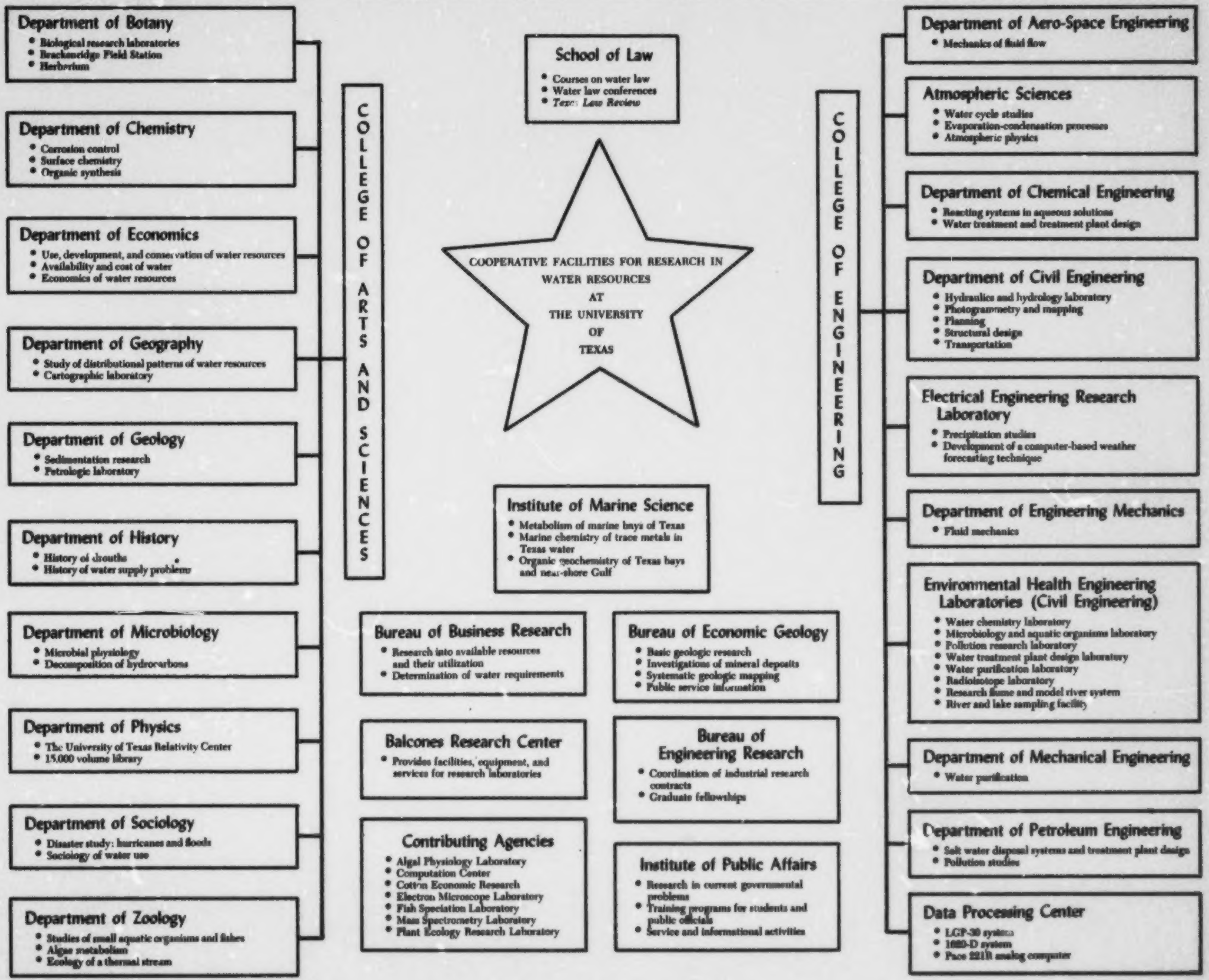

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UPTAKE AND UTILIZATION OF AMINO ACIDS DURING ANAEROBIC DIGESTION

by

Ernest M. Miholits
Joseph F. Malina, Jr.

Final Report
Submitted Under
Grant WP-00083-03
to
Division of Water Supply and Pollution Control
U. S. Public Health Service

CENTER FOR RESEARCH IN WATER RESOURCES
Environmental Health Engineering Research Laboratories
Civil Engineering Department
The University of Texas

EHE-11-6505
CRWR-11

November 1965

Center for Research in Water Resources

THE UNIVERSITY OF TEXAS / ENGINEERING LABORATORIES BUILDING 305 / AUSTIN, TEXAS 78712

Director:
EARNEST F. GLOYNA
Professor of Civil Engineering
(Environmental Health Engineering)

December 23, 1965

U.S. Public Health Service
Division of Water Supply and Pollution Control
Research Grants Section
Washington, D.C.

Gentlemen:

Transmitted herewith is our Final Report which you authorized under research grant WP-00083-03 dated October 1, 1964 through September 30, 1965.

The ~~This~~ report represents a detailed study of the effects of amino acids on the anaerobic sludge digestion process and the effects of the anaerobic environment on the degradation of five selected amino acids. Radiotracer techniques permitted a detailed assay of the distribution of the individual amino acids in the liquid, solid, and gaseous phases.

The purpose of the is to

The control of the amino acid concentration in plant-scale anaerobic digestions systems may lead to a more efficient operation and increased gas production. It is ~~hoped that this~~ report will serve as the basis for a more extensive evaluation of the role of amino acids during anaerobic digestion and for the establishment of the relationships of amino acids to the effective stabilization of wastewater solids. (M)

Respectfully submitted,

Joseph F. Malina, Jr.

Joseph F. Malina, Jr.
Principal Investigator

JFM/vr

Advisory Committee:
Harold C. Bold, *Chairman, Botany Department*
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John R. Stockton, *Director, Bureau of Business Research*

ACKNOWLEDGMENTS

Financial assistance of the Research Grants Section and the Training Grants Section of the Division of Water Supply and Pollution Control of the United States Public Health Service made this study possible.

ABSTRACT

The purpose of this study was to establish the mechanism of anaerobic degradation of amino acids and to evaluate the role of amino acids in the overall metabolism of anaerobic wastewater sludges at 35°C.

Amino acids which are common constituents of wastewater sludges are necessary for the growth and metabolism of the microbial population responsible for the anaerobic stabilization of the organic materials. Hence, the rate of uptake and utilization of amino acids and the gas production rate indicate the total metabolic activity in the digestion unit. An understanding of the incorporation (uptake), utilization, and total turnover of amino acids during anaerobic digestion is necessary to improve the effectiveness of this process in the treatment of wastewater sludges.

Individual radioactive amino acids with the carboxyl carbon labeled were added to samples of sludge which were withdrawn from laboratory-scale anaerobic digestion units. The distribution of the labeled carbon in the gas, sludge solids, and supernatant was evaluated for each of the amino acids.

The results of this investigation indicate that a portion of the amino acids which were introduced to various sludges were adsorbed by the sludge solids at 4°C. However, at 35°C the amino acids were taken up, pooled, and utilized by the microbial population associated with the sludge. Radioactive amino acids with the carboxyl carbon labeled entered into various metabolic reactions which resulted in the production of ammonia, labeled carbon dioxide, and labeled methane gas. Some of the labeled amino acids were in solution as free amino acids; however, no labeled intermediate breakdown products were detected. Low concentrations of amino acids stimulated the production of gas during anaerobic digestion of wastewater sludge.

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CHAPTER 1

INTRODUCTION

Many technical advances in control and operation of the anaerobic digestion process have been made; however, detailed information pertaining to the microbiology, biochemistry, and kinetics of this process is limited. The microbial degradation of wastewater solids during anaerobic digestion results in the conversion of a portion of the organic solids to aqueous compounds which are subsequently utilized by bacteria as a source of energy and nutrients. The major products resulting from anaerobic degradation of organic material are carbon dioxide and methane gas.

Amino acids are common constituents of wastewater sludges and are also released during the degradation and hydrolysis of proteins. Proteins make up about 30 to 50 percent of the weight of organic solids in wastewaters. Therefore, an understanding of the incorporation (uptake), utilization, and total turnover of amino acids during anaerobic digestion is necessary to improve the effectiveness of this process in the treatment of wastewater sludges.

Objectives

The primary objectives of this study included evaluations of:

1) The effects of amino acids on the anaerobic digestion process, and

2) The effects of the anaerobic environment on the incorporation (uptake) and utilization of amino acids during sludge digestion.

Scope

Radioactive amino acids with the carboxyl carbon labeled were added individually to samples of sludge which were withdrawn from laboratory-scale anaerobic digestion units. The amino acids included glycine 1-C-14, L-alanine 1-C-14, L-leucine 1-C-14, DL-methionine 1-C-14, and L-tyrosine 1-C-14. The effects of these compounds on the rate of gas production and on the composition of the gas produced during anaerobic digestion were determined. The distribution of labeled carbon in the gas, sludge solids, and supernatant was also evaluated for each of the amino acids over a 96-hour contact period and at temperatures of 35°C and 4°C. A Warburg respirometer and specially designed flasks were used in these studies.

CHAPTER 2

ANAEROBIC SLUDGE DIGESTION

The anaerobic fermentation (16) process is a biological system used for the treatment and stabilization of organic wastes into innocuous end products. This process consists of two separate stages which take place simultaneously in a continuous digestion operation. The sequential process includes (1) liquefaction of particulate material and hydrolysis of complex molecules to fatty acids and other dissolved intermediate compounds, and (2) gasification, the conversion of dissolved intermediates and compounds to carbon dioxide and methane gas.

The rate-controlling step in the digestion process as this process is employed today is the conversion of the fatty acids to carbon dioxide and methane gas. During liquefaction bacteria secrete extracellular enzymes to convert the organic solids to soluble materials and to hydrolyze complex organic compounds to simpler compounds (e.g., proteins to peptides and amino acids). Facultative and anaerobic bacteria convert the proteins, amino acids, carbohydrates, and fats primarily to the fatty acids, mainly acetic, butyric, and propionic acids. During this stage of digestion the total amount of organic matter in suspension or solution remains essentially unchanged since very little gas is produced. The organic compounds are converted to organic acids and new organic cells (94).

In gasification the methane bacteria convert the soluble organic compounds, such as fatty acids, primarily to carbon dioxide and methane gas. An 80 to 90 percent conversion of fatty acids to carbon dioxide and methane gas can be attained during anaerobic fermentation (94). The amount of gas produced is directly proportional to the amount of organics removed. Tables D-1, D-2, D-3, and D-4 in Appendix D show the content of various constituents of fresh and digested sludge on a dry-weight basis (11,22,70,123).

The fermentation of a simple organic compound, such as an amino acid, can result in fatty acids, carbon dioxide, ammonia, and other products. The fermentation of complex organic compounds, such as carbohydrates, proteins, and fats, results in a multitude of end products which are dependent upon the particular species of acid-forming bacteria involved. The major portion of these end products includes fatty acids (2). This variability of end products with the species of acid-forming bacteria can disturb the equilibrium between the acid-forming bacteria and the methane-forming bacteria and eventually result in an upset digestion process (95,97).

2-1 FACTORS THAT AFFECT ANAEROBIC SLUDGE DIGESTION

Some of the factors which affect sludge digestion are: temperature, pH, detention time, mixing, solids content, sludge composition, microbial population, and combinations of these factors (2,96). The rate of liquefaction proceeds at a higher rate at higher temperatures as indicated by the greater reduction in volatile material and the increase in concentration of volatile or fatty acids (87).

Temperature has a characteristic influence on the rate of gas production. The maximum rate occurred at 32.5°C, an intermediate rate at 52.5°C, and a minimum rate at 42.5°C during studies at these three temperatures only (87). The reduction in volatile material and accumulation of volatile or fatty acids at 42.5°C indicate that the activity of the liquefying organisms is approaching that of the microbial population at 52.5°C (87). The results of other investigations of the effects of digestion of sewage solids above 45°C are not in agreement with the results given above. Heukelekian (69) found that gas yield, volatile matter reduction, and decomposition of nitrogenous substances were greater above 45°C than at lower temperatures. Other investigations show that there were no significant differences in the reduction of volatile material, volume of gas produced, composition of gas, nature of acids produced, and morphology of the organisms in samples of sludges digested at temperatures ranging between 35°C and 60°C (58,62). However, the studies of Golueke (62) show that at 45°C the gas production is lower than at 35°C and 60°C (90). Mixing the digester contents by gas recirculation results in a uniform distribution of microorganisms and nutrients, prevents the accumulation of waste materials at the site of microbial metabolism, and brings carbon dioxide in contact with the microorganisms for subsequent reduction to methane.

Methane bacteria are most active in the pH range of 6.4 to 7.2 (16), and the most efficient methane production during anaerobic digestion occurs in the pH range of 6.5 to 7.8 (122) or 6.7 to 7.4 (95). Liquefaction results in a pH drop from near neutral to about 5.0. This low pH is the result of an accumulation of acetic, propionic, and butyric acids. The subsequent use of these fatty acids by the methane bacteria results in the release of methane and carbon dioxide with a rise in pH to between 6.8 and 7.4 (89). The liquefying organisms are more resistant to changes in the environment than are the gasifying organisms. Liquefaction proceeds at a more rapid rate than gasification; therefore, there may be an accumulation of volatile or fatty acids in the environment (89).

An increase in the detention time up to a maximum of 30 days results in an increase in the percentage of volatile material destroyed.

2-2 DEGRADATION AND METABOLISM OF ORGANIC COMPOUNDS

The organic constituents of sludge: namely, carbohydrates, lipids, proteins, and other organic compounds, are decomposed during anaerobic digestion and provide energy and nutrients for the microbial population. Bacteria can utilize substances for energy and growth only if these metabolites enter the cell. Therefore, it is essential that the particulate material be dissolved and the large organic molecules in sludge be hydrolyzed if these materials are to be used by the microbial population. Extracellular enzymes are excreted by the cells to promote the hydrolysis of these compounds into smaller molecules capable of diffusing through the cell membrane of the bacteria. The amino acids resulting from the hydrolysis of proteins can enter the cell for further degradation or utilization. Many bacteria cannot form the extracellular enzymes required to hydrolyze the proteins unless a sufficient energy supply, such as glucose, is available (31). In addition, a small amount of ammonia nitrogen or amino acid is also required by some microorganisms to initiate the synthesis of extracellular enzymes (120).

The polysaccharides of the carbohydrates are hydrolyzed by extracellular enzymes to form monosaccharides, primarily glucose (112). The monosaccharides pass through the cell membrane for further degradation and utilization.

The lipids are hydrolyzed to fatty acids and glycerol which enter the cell for further degradation and utilization. The long-chain fatty acids are broken down to 2-carbon acids through a process known as beta-oxidation (75,94). The fatty acids are the primary precursors of methane in anaerobic sludge digestion. Approximately 70 percent of the methane produced during anaerobic digestion is formed from acetic acid, while about 30 percent of this gas is formed by the reduction of carbon dioxide (75,94).

The short-chain fatty acids are the major intermediates produced by the acid-forming bacteria from carbohydrates and proteins (95). Acetic acid is the most prevalent fatty acid formed. Up to 75 percent of the methane results from acetic acid during the fermentation of proteins and carbohydrates (95).

2-3 MICROBIOLOGY OF ANAEROBIC SLUDGE DIGESTION

The bacteria responsible for sludge digestion are either facultative bacteria or obligate anaerobic bacteria. The predominant acid producers are various species of *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, Clostridia, Escherichia, and Aerobacter (126). Diplococcus glycinophilus is a bacteria which ferments only the amino acid glycine (26,27,41,124). In addition, species of Clostridium and Lactobacillus have fermented other amino acids to fatty acids.

The methane fermenters are obligate anaerobes and are classified according to Barker (16) in Table 2-1. All of the methane bacteria convert simple organic substances to methane under anaerobic conditions. However, each bacterial species has specific metabolic requirements and can only ferment a relatively restricted group of simple organic compounds.

2-4 BACTERIAL FERMENTATIONS

In bacterial fermentation there is an orderly and controlled dissimilation of the substrate (without molecular oxygen acting as the hydrogen acceptor) in such a manner that energy and building material are made available to the cell.

Fatty acids are intermediate products in the digestion process prior to the conversion to methane and carbon dioxide gas. The anaerobic fermentation of amino acids also yields fatty acids. Therefore, it is necessary to understand the mechanisms involved in the conversion of fatty acids to carbon dioxide and methane gas in order to understand the breakdown of amino acids to gaseous products. The 2-carbon fatty acid, acetate, is the final breakdown product of all fatty acids and the precursor of carbon dioxide and methane gas.

Table 2-1

Classification of Methane Bacteria According to the Compounds Fermented
After Barker (16)

Rod-shaped cells

I. Non-sporulating: *Methanobacterium*

  1. *Mbact. formicicum*: formate, carbon monoxide, hydrogen

  2. *Mbact. propionicum*: propionate

  3. *Mbact. sohngenii*: acetate, butyrate

II. Sporulating: *Methanobacillus*

  1. *Mbac. omelianskii*: primary and secondary alcohols, hydrogen

Spherical cells

I. Cells not in sarcina arrangement: *Methanococcus*

  1. *Mc. mazei*: acetate, butyrate

  2. *Mc. vannielii*: formate, hydrogen

II. Cells in sarcina arrangement: *Methanosarcina*

  1. *Ms. barkerii*: methanol, acetate, carbon monoxide, hydrogen

  2. *Ms. methanica*: acetate, butyrate

2-5 BIOCHEMISTRY OF METHANE FERMENTATIONS

Fatty acids serve as substrates for methane production; the simplest such reaction is the breakdown of formic acid (134,136):

$$4\ HCOOH \longrightarrow CH_4 + 3\ CO_2 + 2\ H_2O$$

The reduction of carbon dioxide with molecular hydrogen (31) also results in methane formation.

$$CO_2 + 4\ H_2 \longrightarrow CH_4 + 2\ H_2O$$

Methane is the only hydrocarbon produced by Methanosarcina methanica, Methanococcus mazei, Methanobacterium sohngenii, and Methanobacterium omelianskii regardless of the number of carbon atoms in the substrate (12,13). When ethanol substrate was used, there was a quantitative dehydrogenation of the alcohol to acetic acid, along with the reduction of some carbon dioxide (12,13).

$$2\ C_2H_5OH + 2\ H_2O + CO_2 \longrightarrow 2\ CH_3COOH + CH_4 + 2\ H_2O$$

The acetic acid is then converted to methane.

$$CH_3COOH + 2\ H_2O + CO_2 \longrightarrow 2\ CO_2 + CH_4 + 2\ H_2O$$

The results of studies on the fermentation of acetate indicate that methane may arise from two sources, carbon dioxide and the methyl group of acetate; however, the source of this product depends on the substrate available and species of organisms present.

All fermentations have one property in common, the major energy yielding reactions are dehydrogenations in which substances other than molecular oxygen are the ultimate hydrogen and electron acceptors.

The fermentation of a substrate containing unlabeled acetic acid and labeled carbon dioxide by a heterogeneous anaerobic culture showed that (131):

1) 86 to 94 percent of the methane was derived from the methyl group of acetic acid;

2) 96 to 98 percent of the carbon dioxide was derived from the carboxyl group of acetic acid; and

3) 3 to 11 percent of the methane was derived from carbon dioxide after the fermentation had progressed for a period from 5 to 30 days.

Methane originated from the methyl group of acetate and the carbon dioxide originated with the carboxyl group of acetate during fermentation by a pure culture of Methanosarcina (132,133). Methane fermentation of acetate differs from that of most other substrates in that carbon dioxide reduction is a minor reaction.

$$C^{14}H_3COOH + CO_2 + 2\,H_2O \longrightarrow 2\,CO_2 + C^{14}H_4 + 2\,H_2O$$

Most, if not all, of the methane is derived from acetate while almost none is derived from carbon dioxide under the above experimental conditions. Labeled carbon dioxide was reduced to methane and some of the carbon dioxide was assimilated by the cells (14). In a methane fermentation of acetic acid in the presence of labeled carbon dioxide by Methanococcus, the methane was almost completely unlabeled (131).

The fermentation of acetate substrates, in which both carboxyl and methyl carbons were labeled, by Methanococcus produced the following results (24,117,118,131):

$$C^{14}H_3COOH + CO_2 + 2\,H_2O \longrightarrow 2\,CO_2 + C^{14}H_4 + 2\,H_2O$$

$$CH_3C^{14}OOH + CO_2 + 2\,H_2O \longrightarrow C^{14}O_2 + CO_2 + CH_4 + 2\,H_2O$$

$$CH_3COOH + C^{14}O_2 + 2\,H_2O \longrightarrow (1-x)C^{14}O_2 + CO_2 + x\,C^{14}H_4 + CH_4 + 2\,H_2O$$

where $x \ll 1$

2-6 FERMENTATION OF PROPIONATE, BUTYRATE, AND VALERATE

A discussion of the fermentation of propionate, butyrate, and valerate is important to this study because these fatty acids are products of liquefaction, constituents of digesting sludge, and precursors of acetate.

Fermentation of Propionate

The fermentation of propionate by Methanobacterium propionicum proceeds according to the equation (132):

$$4\ CH_3CH_2COOH + 8\ H_2O \longrightarrow 4\ CH_3COOH + 4\ CO_2 + 24\ H$$

$$3\ CO_2 + 24\ H \longrightarrow 3\ CH_4 + 6\ H_2O$$

$$4\ CH_3CH_2COOH + 2\ H_2O \longrightarrow 4\ CH_3COOH + CO_2 + 3\ CH_4$$

The overall equation for the fermentation of unlabeled propionate and labeled carbon dioxide is as follows:

$$4\ CH_3CH_2COOH + 2\ H_2O + 4\ C^{14}O_2 + 24\ H \longrightarrow$$

$$4\ CH_3COOH + CO_2 + C^{14}O_2 + 3\ CH_4 + 3\ C^{14}H_4 + 6\ H_2O$$

However, the fermentation of carboxyl labeled propionate by Methanobacterium propionicum yields:

$$CH_3CH_2C^{14}OOH + 2\ H_2O \longrightarrow CH_3COOH + C^{14}O_2 + 6\ H$$

Fermentation of Butyrate

The fermentation of unlabeled butyrate in the presence of labeled carbon dioxide by Methanobacterium suboxydans proceeds as follows:

$$2\ CH_3CH_2CH_2COOH + 2\ H_2O + C^{14}O_2 \longrightarrow 4\ CH_3COOH + C^{14}H_4$$

Almost all of the methane was derived from carbon dioxide. This organism was unable to ferment acetate (132).

Fermentation of Valerate

The fermentation of unlabeled valerate in the presence of labeled carbon dioxide by Methanobacterium suboxydans proceeds as follows:

$$2\ CH_3CH_2CH_2CH_2COOH + 2\ H_2O + C^{14}O_2 \longrightarrow$$

$$2\ CH_3CH_2COOH + 2\ CH_3COOH + C^{14}H_4$$

Practically all of the methane produced in this fermentation was derived from the carbon dioxide (132).

The fermentation of valerate (labeled in the number three carbon position) by Methanobacterium suboxydans proceeds as follows:

$$2\ CH_3CH_2C^{14}H_2CH_2COOH + 2\ H_2O + CO_2 \longrightarrow$$

$$2\ CH_3CH_2C^{14}OOH + 2\ CH_3COOH + CH_4$$

All of the labeled carbon of valerate was found in the carboxyl carbon of propionate, thus substantiating the beta-oxidation of fatty acids. The absence of carbon-labeled carbon dioxide and methane is evidence that no carbon dioxide was formed from propionate, thus indicating the inability of Methanobacterium suboxydans to oxidize fatty acids beyond the C_3 stage.

2-7 SUMMARY OF ANAEROBIC SLUDGE DIGESTION

In conclusion, isotopic data with methane-fermenting organisms have shown that the fermentation of fatty acids proceeds according to a scheme called Knoop's "β-oxidation" theory (66). Briefly, this theory involves a split between the number 2 and number 3 carbon of a fatty acid, the addition of a water molecule, and the production of one molecule of acetic acid and one molecule of a fatty acid with two carbons less than originally (i.e., the loss of a $(CH_2)_2$ group) (94).

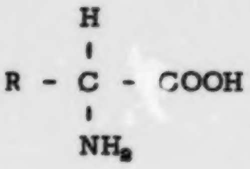
The study of the fate of carbon compounds in anaerobic fermentation is the study of the breakdown of acetate, since this is the final product before dissociation into carbon dioxide and methane gas; however, propionate is an exception.

According to McCarty (94) acetic acid is the major acid produced in the degradation of the fatty acids, carbohydrates, and proteins under all digester conditions. Propionic acid was also present in major proportions in the degradation of carbohydrates and proteins.

CHAPTER 3

AMINO ACIDS

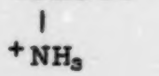
An amino acid is a chemical compound which contains amino and carboxyl groups. Most of the natural amino acids (the alpha amino acids) are described by the general structure:



$$R - \underset{\underset{\displaystyle NH_2}{|}}{\overset{\overset{\displaystyle H}{|}}{C}} - COOH$$

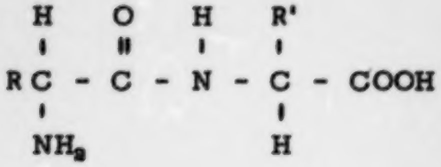
The amino group is attached to the number two carbon (the alpha carbon), and the carboxyl carbon is the number one carbon. Exceptions to these generalizations include amino acids which have (1) more than one amino or carboxyl group, (2) imino groups, (3) other types of acid groups, or (4) the amino group attached to other than the number two carbon. There are 22 amino acids which are said to occur frequently in protein hydrolyzates. A classification of these amino acids is shown in Table 3-1.

Proteins consist of large numbers of α-amino acids linked together by the elimination of water at each link. Amino acids in neutral solution are not in the neutral form $RCHNH_2COOH$ but in the doubly charged form,



$$R\underset{\underset{\displaystyle {}^{+}NH_3}{|}}{C}HCOO^{-}$$

The resulting protein molecules are highly polar, and most of these compounds are soluble in water but not in organic solvents (150). Proteins differ in number of amino acids, their order or combination, and the structure resulting from their arrangement in peptide linkages.



$$R\underset{\substack{|\\NH_2}}{\overset{\substack{H\\|}}{C}} - \overset{\substack{O\\\|}}{C} - \overset{\substack{H\\|}}{N} - \underset{\substack{|\\H}}{\overset{\substack{R'\\|}}{C}} - COOH$$

Peptide Linkage

Amino acids are found in domestic wastewater sludges (115,154, 164).

3-1 AMINO ACIDS AND THE BACTERIAL CELL

Microorganisms require nitrogen, carbon, and energy to carry out their metabolic activities. These requirements for some bacteria can be satisfied by amino acids, $RCHNH_2COOH$ (1,104). Many organisms are not capable of synthesizing all of the required amino acids and must obtain these compounds from the environment. The source of amino acids in nature is through synthesis by living cells.

The amino acids are essential to microbial metabolism because these compounds are required in (98):

1) The formation of new proteins or the incorporation of the amino

Table 3-1

Amino Acids in Proteins

Category of Amino Acids	Amino Acids in Each Group or Category
A) Aliphatic	
1) Monoaminomonocarboxylic	Glycine, alanine, isoleucine, leucine, valine
2) Hydroxymonoaminomonocarboxylic	Serine, threonine
3) Monoaminodicarboxylic	Glutamic acid, aspartic acid
4) Monoaminodicarboxylic amides	Asparagine, glutamine
5) Diaminomonocarboxylic	Arginine, lysine
6) Sulfur containing	Cysteine, cystine, methionine
B) Aromatic	Phenylalanine, tyrosine
C) Heterocyclic	Tryptophan, histidine, proline, hydroxyproline

acids into existing proteins, and

2) The conversion of amino acids to other products, such as
    a) allantoin, uric acid, creatine, and other products,
    b) other amino acids, and
    c) peptides, vitamins, nucleic acids, and other compounds.

Amino acids are synthesized by living cells by means of the metabolic pathways shown in Fig. 3-1. The cell converts ammonia into the amino group (112). These two pathways are the only identified systems by which living cells can convert nitrogen from the non-amino form into the amino form (112). These pathways are commonly called the fumarate gateway and the ketoglutarate gateway (112). The more important reactions of alpha amino acids are also summarized in Fig. 3-1.

Most bacteria are not capable of synthesizing all of the amino acids that are required for their metabolism. Streptococcus faecalis requires at least 15 of the amino acids in the substrate; Streptococcus salivarus requires only two amino acids (160).

If an organism cannot synthesize a necessary amino acid, the amino acid must be derived from the environment. A good source of amino acids is protein; however, most protein molecules are too large to pass through the cell membrane (31). Proteins are hydrolyzed to form free amino acids, peptides, and other protein fragments by extracellular enzymes secreted by the microorganisms.

Several common types of reactions in which microorganisms may participate in the presence of amino acids are (34,63,150):

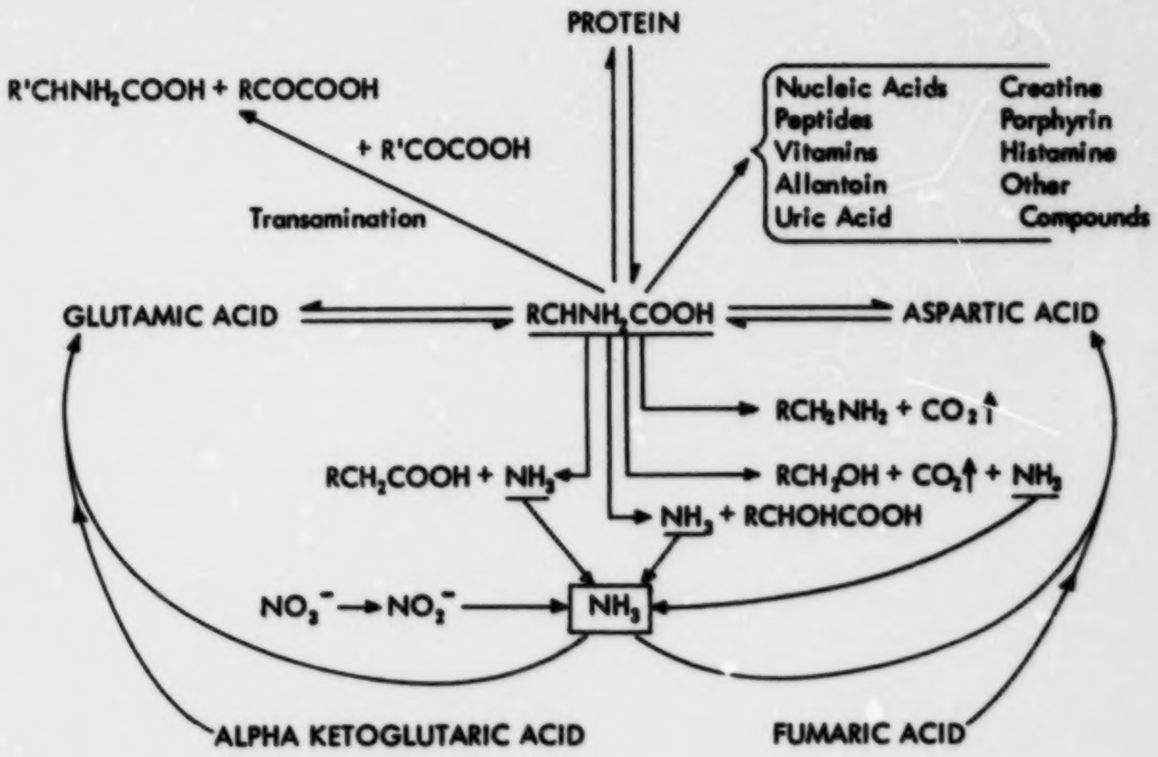

PROTEIN
R'CHNH$_2$COOH + RCOCOOH
+ R'COCOOH
Transamination
Nucleic Acids
Peptides
Vitamins
Allantoin
Uric Acid
Creatine
Porphyrin
Histamine
Other Compounds
GLUTAMIC ACID
RCHNH$_2$COOH
ASPARTIC ACID
RCH$_2$NH$_2$ + CO$_2$↑
RCH$_2$COOH + NH$_3$
RCH$_2$OH + CO$_2$↑ + NH$_3$
NH$_3$ + RCHOHCOOH
NO$_3^-$ → NO$_2^-$
NH$_3$
ALPHA KETOGLUTARIC ACID
FUMARIC ACID

Fig. 3-1. Some Reactions of Alpha Amino Acids

1) Formation of keto acids (deamination).

$$RCHNH_2COOH + 1/2\ O_2 \longrightarrow NH_3 + RCOCOOH$$

2) Formation of hydroxyacids (deamination).

$$RCHNH_2COOH + H_2O \longrightarrow NH_3 + RCHOHCOOH$$

3) Formation of fatty acids (deamination).

$$RCHNH_2COOH + 2\ H \longrightarrow NH_3 + RCH_2COOH$$

4) Formation of amines (decarboxylation).

$$RCHNH_2COOH \longrightarrow CO_2 + RCH_2NH_2$$

5) Formation of alcohols (deamination and decarboxylation).

$$RCHNH_2COOH + H_2O \longrightarrow NH_3 + CO_2 + RCH_2OH$$

6) Direct utilization for the formation of proteins.

3-2 AMINO ACID REQUIREMENTS OF MICROORGANISMS

The amino acid requirements of bacteria vary over a broad range from those organisms which can synthesize all of the component amino acids (e.g., Escherichia coli) to those which exhibit highly complex requirements (e.g., Leuconostoc mesenteroides). The amino acid requirements of a few bacteria are listed in Table 3-2. Albritton (1) has shown that at least one species of bacteria can exist entirely on each of the common amino acids as the sole nitrogen source.

Factors which affect the growth responses to amino acids are (114):

1) Molecular structure and concentration of the amino acid,
2) The presence and concentration of other amino acids in the medium,

Table 3-2

Amino Acid Requirements of Several Bacteria

Amino Acid	Lactobacillus arabinosus (100)	Streptococcus lactis (111)	Lactobacillus casei (74)	Streptococcus faecalis (130)	Leuconostoc mesenteroides (64)	Reiter treponema (140)
Alanine	-	-	(+)	+	(+)	-
Arginine	(+)	+	+	+	+	+
Aspartic Acid	(+)	-	+	+	+	+
Cystine	+	-	+	(+)	+	+
Glutamic Acid	+	-	+	+	+	+
Glycine	-	-	-	+	+	-
Histidine	(+)	(+)	(+)	-	+	+
Isoleucine	+	+	(+)	+	+	+
Leucine	+	+	+	+	+	+
Lysine	+	(+)	(+)	+	+	+
Methionine	(+)	+	(+)	-	+	+
Phenylalanine	(+)	(+)	+	-	+	+
Proline	-	-	-	-	+	(b)
Serine	-	(+)	+	+	+	(+)
Threonine	(+)	-	(+)	+	+	+
Tryptophan	+	-	+	+	+	+
Tyrosine	(+)	-	+	(+)	+	(+)
Valine	+	+	+	-	+	+

Note: + essential; - nonessential; (+) accessory; (b) may substitute for glutamic acid.

3) The oxygen tension in the substrate,
4) The amount of vitamins, trace elements, and other growth factors,
5) Carbohydrates in the medium,
6) Antagonists in the medium, and
7) The duration of the test period.

The rate of incorporation of an amino acid, such as glutamic acid, was different when glutamic acid was the only amino acid present in the medium from that when 17 other common amino acids were present (57). When glutamic acid alone was present, Straphylococcus aureus incorporated only a fraction of the amino acid (55).

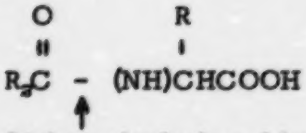
The study of the uptake and utilization of individual amino acids is a very complex topic. The concentration of amino acids and other organic constituents in anaerobic sludges is quite variable. The specific pattern by which amino acids are degraded in a variable environment containing heteorogeneous populations of organisms competing for nutrients which are necessary for growth and energy may be much different from the pathways reported in studies in which defined substrates and single species were used.

3-3 AMINO ACID METABOLISM

Proteins can serve as sources of carbon, nitrogen, and energy for the growth of many species of bacteria. Only a few microbial species are capable of degrading the more native proteins (i.e., proteins in the state in which they exist in the intact cell) into simpler compounds that could be utilized within the cell.

In general, native proteins are attacked by bacteria only when sufficient amounts of simpler nitrogenous substances which will support the initiation of growth are not present. After growth has started, extracellular proteinases are produced by the cells and may be found in the medium. However, if utilizable carbohydrates are present in the medium, the formation of proteinases is inhibited (106).

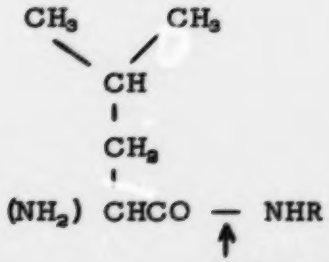
Proteins are first hydrolyzed by proteolytic enzymes (e.g., proteinases) or chemical agents into simpler units of peptones and polypeptides; continued hydrolysis leads to the liberation of the component amino acids (101). Most bacteria possess the enzyme peptidases. The carboxypeptidases attack the carboxyl end of the peptide chain (31,101).



$$R_2\overset{\overset{\displaystyle O}{\|}}{C} - (NH)\overset{\overset{\displaystyle R}{|}}{C}HCOOH$$

Linkage hydrolyzed by
carboxypeptidase

The aminopeptidases catalyze the hydrolysis of peptide linkages adjacent to free α-amino groups of peptides (31,101).



Linkage hydrolyzed by
amino peptidase

The end result of the activity of proteinases and peptidases is the release of amino acids into the substrate. The amino acids can penetrate the cell membrane and can be utilized by the cell for metabolic functions.

Amino acids are used by the cell for synthesis and as sources of oxidative energy. The removal of the amino group from amino acids frequently leads to the formation of compounds such as pyruvic acid and α-ketoglutaric acid. An example of this reaction is as follows:

$$\underset{\text{alanine}}{CH_3CH_2(NH_2)COOH} + 1/2\ O_2 \longrightarrow \underset{\text{pyruvic acid}}{CH_3COCOOH} + NH_3 + H^+$$

Decarboxylation (removal of a CO_2 group) of amino acids is carried out by many species of bacteria; sometimes both decarboxylation and deamination occur. In other instances, the amino acid is split before it is further dissimilated (46). Deamination (removal of a NH_2 group) of an amino acid occurs most readily in an alkaline medium and decarboxylation in an acidic medium. Escherichia coli forms glutamic dehydrogenase when grown at pH 5 and glutamic deaminase when grown at pH 8 (52). The formation of the appropriate enzyme is thus induced by the pH of the medium. The ability to form different enzymes allows the organism to achieve some degree of internal pH stabilization and to have the ability to get nutrients. Deamination in an alkaline medium results in the production of an acid which tends to lower the pH.

There are four general mechanisms for deamination:

1) Oxidative deamination of an amino acid is a Strickland reaction (31, 142) wherein a second amino acid acts as the oxidant. This reaction is anaerobic and involves the organism Clostridium.

$$\underset{\text{glycine}}{2\,CH_2(NH_2)COOH} + \underset{\text{alanine}}{CH_3CH(NH_2)COOH} + 2\,H_2O \longrightarrow$$

$$3\,CH_3COOH + 3\,NH_3 + CO_2$$

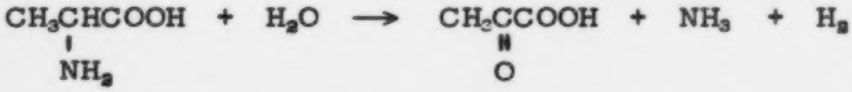
2) Reductive deamination leads to the formation of saturated fatty acids. This anaerobic reaction is common among the organisms of the genus Clostridium (150).

$$RCH(NH_2)COOH + 2\,H \longrightarrow RCH_2COOH + NH_3$$

3) Desaturative deamination leads to the formation of an unsaturated acid according to the equation (150):

$$RCH_2CH(NH_2)COOH \longrightarrow RCH = CHCOOH + NH_3$$

4) Dehydrogenation deamination leads to the formation of a keto acid, ammonia, and hydrogen.



$$CH_3\underset{\underset{NH_2}{|}}{C}HCOOH + H_2O \longrightarrow CH_3\underset{\underset{O}{\|}}{C}COOH + NH_3 + H_2$$

alanine

Alanine thus serves as a donor of hydrogen. In the Strickland reaction, alanine is the hydrogen donor and glycine the hydrogen acceptor.

Decarboxylation of amino acids occurs most frequently in acid solutions (see Table 3-3) and leads to the production of amines (31).

$$\underset{\text{lysine}}{(NH_2)CH_2(CH_2)_3CH(NH_2)COOH} \longrightarrow \underset{\text{cadaverine}}{(NH_2)CH_2(CH_2)_3CH_2(NH_2)} + CO_2$$

Keto acids may be reaminated and incorporated into protein or may undergo degradative reactions which ultimately yield carbon dioxide and water (103).

The end products of many reactions of amino acids are similar to those products resulting from the oxidation of fats and carbohydrates; however, nitrogen is released as ammonia (150). Certain amino acids can, under suitable conditions, lead to the formation of carbohydrates during microbial metabolism. Deamination of aspartic acid, alanine, and glutamic acid yields α-keto acids which are intermediates in the metabolism of carbohydrates (103).

3-4 TRANSFER OF AMINO ACIDS INTO CELLS

The uptake of an amino acid by a cell is affected by the concentration of the amino acid in the surrounding medium, the enzymatic activity of the system which stimulates the transfer of the amino acid into the cell, and the cellular reactions which serve to utilize the amino acid.

Table 3-3

Amino Acid Decarboxylation
Typical Examples (47)

Amino Acid	Organism	Optimum pH	Product
Lysine	B. cadaveris	6.0	Cadaverine
Arginine	E. coli	5.2	Agmatine
Histidine	Cl. welchii	4.5	Histamine
Ornithine	Cl. septicum	5.5	Putrescine
Tyrosine	Sc. fecalis	5.5	Tyramine
Glutamic acid	Cl. welchii	5.5	α-Aminobutyric acid
Aspartic acid	Cl. welchii	-	β-Alanine
Tyrosine	Microorganisms	-	Tyramine (104)
Leucine	Microorganisms	-	3 Methybutylamine (104)

The evaluation of the uptake of amino acids by bacteria is complicated by the simultaneous occurrence of other metabolic reactions involving amino acids, including degradation reactions and incorporation of amino acids during synthesis of protein. Roberts and his co-workers concluded that the bacterial uptake of amino acids and other nutrients may be explained by simple diffusion of these substances into the "water spaces" in the cell and the concentration of the specific compound in the "water spaces" is the same as in the medium (120).

Other workers (79) have shown that Escherichia coli was able to accumulate amino acids against a concentration gradient. Internal concentrations of lysine in cells of Streptococcus faecalis were found to be considerably greater than the surrounding fluid (50,51). This internal pooling of lysine indicates that an absorption process may be taking place, but lysine was still able to pass easily into and out of the cells. Escherichia coli was shown to concentrate C^{14}-valine in the cells to a level 1000 times as great as that of the medium (32). The valine already taken up could be competitively displaced by leucine or isoleucine; however, the uptake was specific for L-valine and proceeded more rapidly than the incorporation into protein.

Glutamic acid can be readily exchanged between the "water space" of cells of Streptococcus faecalis and Straphylococcus aureus and the medium (48,50,52). However, in the presence of glucose, glutamic acid accumulation occurred.

In studies of amino acid assimilation or uptake by various species of bacteria, the gram-positive bacteria were found to concentrate amino acids such as lysine and glutamic acid within the cells from the external environment, while gram-negative bacteria were unable to do so (20,48, 49,149). When the organism *Streptococcus faecalis* was suspended in a solution of lysine, the internal concentration was 2 to 20 times the external concentration (49). With glutamic acid the ratio was 50 to 60 times. Lysine is able to diffuse directly into the cell, but glutamic acid requires an external energy source. The intracellular concentration of glutamic acid in *Micrococcus pyogenes* was 400 times as high as that in the suspension medium. An energy source was required for *Streptococci* if glutamic acid was to leak out of the cell, but for *Staphylococci* an energy source prevented leakage of glutamic acid from the cell into the suspension medium.

The pooling of amino acids by bacteria is such that these solutes are either retained in the free state or combined with some non-diffusible component of the cell. The first condition implies the presence of a semipermeable membrane, and the second condition requires a membrane that allows the free diffusion of the amino acids but retains the non-diffusible complex.

Escherichia coli incorporate ions and low molecular weight organic compounds such as amino acids (120). These amino acids are not free in the cell but are absorbed in larger molecules. The ions and organic compounds can also diffuse out of the cell if they are not rapidly utilized or if they are not bound to cellular components. Intermediates of metabolism are held within the cell in union with co-enzymes or other carriers of large molecular size. An equilibrium is established between the medium and the cells.

The cell wall is not responsible for the uptake or retention of pooled lysine in Micrococcus lysodeikticus; the protoplast membrane apparently controls this process (20).

3-5 BACTERIAL PERMEABILITY AND POOLS

Selective pooling is a major regulatory mechanism in the metabolic activity of bacteria (20). If an amino acid is to be utilized for growth and reproduction of cells, it is necessary that the amino acid, or its breakdown products, be able to enter the bacterial cell (112). The cell membrane controls the passage of dissolved substances into and out of the cell and may also act as a secretory organ involved in the formation of the cell wall and slime layer (31).

The major function of the cell membrane is the maintenance of a relatively isolated internal environment in the cell (31). In an actively growing cell, several hundred thousand molecules of glucose may be utilized per second; thus, the rate of transfer across the cell membrane is very high (31).

The cytoplasmic membrane is the surface structure primarily responsible for the osmotic properties of the cell (33,105,158). The significance of both the cell wall and cell membrane on bacterial permeability and pooling of lysine by Micrococcus lysodeikticus was evaluated and the results of this study (20) indicate:

1) Amino acids are pooled in a free state within a semipermeable membrane;
2) A large quantity of the total uptake was non-pooled lysine which was localized and appreciably concentrated in the cell wall region;
3) A portion of this non-pooled lysine was adsorbed by the cell walls;
4) This diffusional uptake and adsorptive concentration by the cell wall causes the cells of Micrococcus lysodeikticus to take up considerably more lysine than that found in the free internal pool;
5) The internal free amino acid pool is the source of the amino acid that is incorporated into the chemical transformation of the cell;
6) Free intracellular amino acids equilibrate with extracellular amino acids in a different manner in gram-positive organisms from that in gram-negative organisms; and
7) Uptake by the cell wall of gram-positive and gram-negative organisms are not the same because the thickness and chemical composition of the walls are different.

The cell walls of gram-positive and gram-negative bacteria differ greatly in their lipid content. The cell walls of gram-negative bacteria contain 11 to 22 percent by weight of lipids while only 1 to 4 percent of the weight of the cell walls of gram-positive bacteria is lipids. A complete complement of amino acids is contained in the cell walls of gram-negative bacteria; however, in the cell walls of gram-positive bacteria few amino acids are found (57).

The total quantity of low weight molecular compounds that may be extracted from the bacterial cell under conditions that do not degrade macromolecules is defined as the "pool" (21). The formation of a pool allows a cell to provide nutrients at the sites of synthesis in high concentrations, although the concentration of nutrients in the environment is low.

Britten (21) studied the pool in aerobic cultures of Escherichia coli. The results of this investigation were:

1) Passage through the pool appears to be an obligate step for incorporation of an exogenous amino acid into protein.
2) Amino acids present in the pool are incorporated into protein at random, regardless of the length of time they have been in the pool.
3) Peptides do not appear to be intermediates in protein synthesis.
4) An energy source (such as glucose) is required for pool formation to occur at normal rates but is not required for maintenance of the pool for relatively long periods.
5) Specific pool formation mechanisms exist for each amino acid or group of structurally similar amino acids.
6) For any given amino acid, there appears to be a maximum pool size (or saturation value) at large external concentrations.
7) Any damage to the cell's integrity or even mild treatments (for a bacterial cell), such as osmotic shock, lead to loss of the pool.
8) Exchange between pool and external amino acids occurs at a high rate, not only when there is a steady flow through the pool but also in the absence of glucose or at 0°C when the flow through the pool is strongly suppressed (conditions which also suppress pool formation).

The free amino acid pool may not be an exact measure of the available supply of amino acids for the synthesis of new protein molecules. Preexisting proteins may break down to their constituent amino acids and replenish the pool by an internal device. This condition was shown in starvation experiments in which the glutamic acid content in the internal pool was reduced by 80 percent but the enzyme synthesizing ability was reduced by only 37 percent (65). As the starvation process progressed, the proteins which broke down easily contributed to the free amino acid pool, thus continuing the ability of the cells to synthesize enzymes. The prolongation of the starvation process resulted in a recycling of the amino acids in the pool with the proteins in the cell and the eventual trapping of the amino acids in the least labile proteins, which are the least capable of supplying free amino acids to the pool for new enzyme formation. The ability of bacteria to synthesize new enzyme molecules is strongly dependent upon the availability of an adequate supply of amino acids. Free amino acids are the predominant source of nitrogen in the formation of new enzyme molecules. No other amino acid-independent mechanism has been found which transforms a pre-existing complex protein precursor into active enzyme (65).

3-6 ANAEROBIC REACTIONS OF AMINO ACIDS

In the absence of oxygen, amino acids may be degraded to yield fatty acids, ammonia, carbon dioxide, and other products. Degradation reactions of glycine, alanine, leucine, methionine, and tyrosine are subsequently discussed.

Glycine Fermentations

Fermentation of glycine by pure cultures of Diplococcus glycinophilus may be described by the following idealized equation (26,27,41,124):

$$4\ CH_2(NH_2)COOH \ + \ 2\ H_2O \longrightarrow 3\ CH_3COOH \ + \ 2\ CO_2 \ + \ 4\ NH_3$$

The results of studies in which the substrate contained carbon-labeled glycine, carbon dioxide, and acetic acid indicate that (15):

> 1) Approximately 75 percent of the methyl carbon and 54 percent of the carboxyl carbon of acetic acid are derived from the methyl carbon of glycine;
>
> 2) One of the main reactions during the fermentation of glycine is a condensation between two molecules of glycine through their methyl groups resulting in the conversion of the terminal carbons to carbon dioxide and the oxidation of the two central carbons to acetic acid. Data indicate that from 90 to 95 percent of the carbon dioxide is derived from the carboxyl carbon of glycine, the remainder from the methyl carbon. The small amount (5 to 10 percent) of carbon dioxide derived from the methyl carbon indicates the small extent of glycine oxidation; and
>
> 3) At least 6 percent of the methyl carbon and 38 percent of the carboxyl carbon of acetate originate from carbon dioxide. The direct reduction of glycine to acetic acid is a minor reaction.

If hydrogen gas is formed, more carbon dioxide and less acetic acid are formed. Moreover, the idealized equation represents the decomposition of glycine only in stationary cultures where the gas space in contact with the medium is relatively small (27).

If a 10-ml cell suspension and a 90-ml gas space are shaken continuously, the equation becomes (27):

$$10\ CH_2(NH_2)COOH + 9.12\ H_2O \longrightarrow 10\ NH_3 + 5.26\ CH_3COOH + 9.3\ CO_2 + 8.6\ H_2$$

The optimum pH for Diplococcus glycinophilus, a gram-positive, obligate anaerobic organism, is 7.2.

Micrococcus anaerobius and Micrococcus variabilis also ferment glycine in accordance with the idealized equation illustrated for Diplococcus glycinophilus (41). Glycine can also be used by a number of anaerobic bacteria as the hydrogen acceptor in a coupled oxidation-reduction reaction between pairs of amino acids (i.e., a Strickland reaction) (135,136,137).

$$CH_2(NH_2)COOH + 2\,H \longrightarrow CH_3COOH + NH_3$$

The anaerobic organisms Clostridium cylindrosporum (27) and Clostridium kluyveri (151) were found to fix large amounts of labeled carbon dioxide into glycine. The carboxyl group of the glycine isolated from the Clostridium organisms contained practically all of the carbon-14 incorporated into the molecule.

Alanine Fermentations

The equation which describes the fermentation of alanine by Clostridium propionicum may be written as (26,27,61):

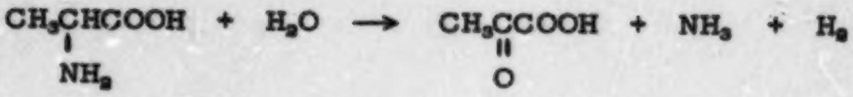
$$3\,CH_3CH(NH_2)COOH + 2\,H_2O \longrightarrow 2\,CH_3CH_2COOH + CH_3COOH + CO_2 + 3\,NH_3$$

Certain anaerobic microorganisms cause mutual oxidation-reduction between two amino acids resulting in the deamination of both amino acids.

The reaction (Strickland reaction) requires the presence of both an amino acid that can accept hydrogen and one that can serve as a hydrogen donor. The amino acids which serve as hydrogen donors are: L-aspartic acid, L-leucine, L-isoleucine, L-valine, L-alanine, L-phenylalanine, L-cysteine, L-serine, L-histidine, and L-glutamic acid. The amino acids which serve as hydrogen acceptors are: glycine, L-ornithine, L-proline, L-hydroxyproline, L-arginine, and L-tryptophan. Such reactions can provide the sole source of energy for *Clostridium sporogenes* and several other bacteria (27,142,143,144). Most of the organisms that catalyze the Strickland reaction belong to the *Clostridiae* family, but not all *Clostridia* are active (142,143,144,163). In the presence of both a hydrogen acceptor and a hydrogen donor, ammonia and carbon dioxide are evolved. The step by step reaction between glycine and alanine is shown on the following page (30).

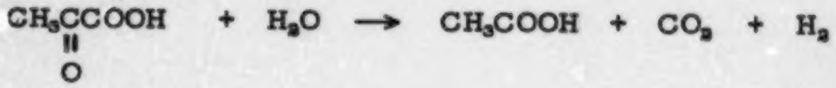
The primary product of reactions involving alanine is pyruvic acid, a carbon compound of metabolic importance and readily available as an energy source for growth of *Escherichia coli* (37). Alanine can serve as a sole nitrogen source and support good growth of *Escherichia coli*. The recognized deaminases of *Escherichia coli* all yield α-keto acids of prime metabolic importance.

Yeast extract or its equivalent is necessary for *Clostridium propionicum* to ferment alanine. But growth on yeast extract alone will be very poor unless a readily fermentable compound such as alanine or one of the related compounds (lactate, pyruvate, serine, or threonine) is present (26).



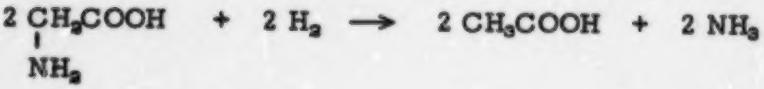
$$\underset{\displaystyle NH_2}{CH_3\underset{|}{C}HCOOH} + H_2O \longrightarrow CH_3\underset{\displaystyle O}{\underset{\|}{C}}COOH + NH_3 + H_2$$

alanine — pyruvic acid



$$CH_3\underset{\displaystyle O}{\underset{\|}{C}}COOH + H_2O \longrightarrow CH_3COOH + CO_2 + H_2$$

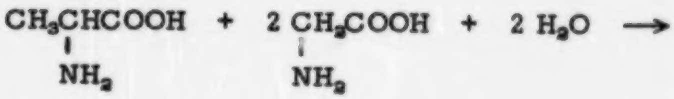
pyruvic acid — acetic acid



$$2\ \underset{\displaystyle NH_2}{\underset{|}{C}H_2COOH} + 2\ H_2 \longrightarrow 2\ CH_3COOH + 2\ NH_3$$

glycine — acetic acid

Sum:



$$\underset{\displaystyle NH_2}{CH_3\underset{|}{C}HCOOH} + 2\ \underset{\displaystyle NH_2}{\underset{|}{C}H_2COOH} + 2\ H_2O \longrightarrow$$

alanine — glycine

$$3\ CH_3COOH + 3\ NH_3 + CO_2$$

acetic acid

Leucine Fermentation

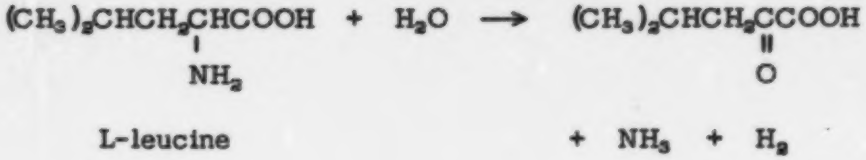
The only evidence of the anaerobic degradation of leucine by microorganisms is by means of a mutual oxidation-reduction reaction between two amino acids (the Strickland reaction) (104). L-leucine participates as a hydrogen donor in the Strickland reaction which is catalyzed by the organism Clostridium sporogenes and some other species of Clostridium (142,143,144,163). As an example, the complete anaerobic reaction between L-leucine and L-proline (a hydrogen acceptor) is shown on the following page (30,72,91,110).

Some microorganisms can decarboxylate leucine to yield amines and also participate in transamination reactions. Leucine is decarboxylated enzymatically to 3-Methylbutylamine by Proteus vulgaris at an optimum pH of 7 (43,68,86,147). Claviceps purpurea also decarboxylated leucine (67).

Leucine is also involved in an α-ketoglutarate-amino acid transamination which results in the formation of glutamate and involves either Pseudomonas fluorescens, Escherichia coli, or Leuconostoc arabinosus (44, 102). Glutamate then can serve as a hydrogen donor in the anaerobic Strickland reaction. Pseudomonas fluorescens is an anaerobic organism and Escherichia coli is a facultative anaerobe. Both organisms are found in anaerobic wastewater sludge (126).

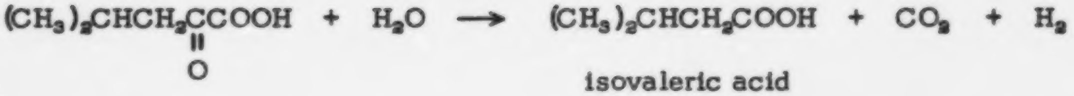
Methionine Fermentations

Methionine is fermented by certain strains of Pseudomonas to yield ammonia, α-keto butyric acid, and methyl mercaptan (77). Methionine



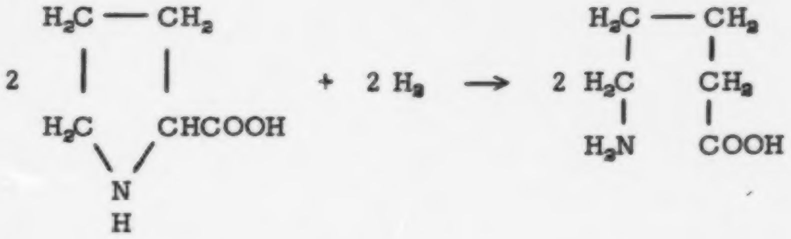
$$(CH_3)_2CHCH_2\underset{NH_2}{\underset{|}{C}}HCOOH + H_2O \longrightarrow (CH_3)_2CHCH_2\underset{O}{\underset{\|}{C}}COOH + NH_3 + H_2$$

L-leucine



$$(CH_3)_2CHCH_2\underset{O}{\underset{\|}{C}}COOH + H_2O \longrightarrow (CH_3)_2CHCH_2COOH + CO_2 + H_2$$

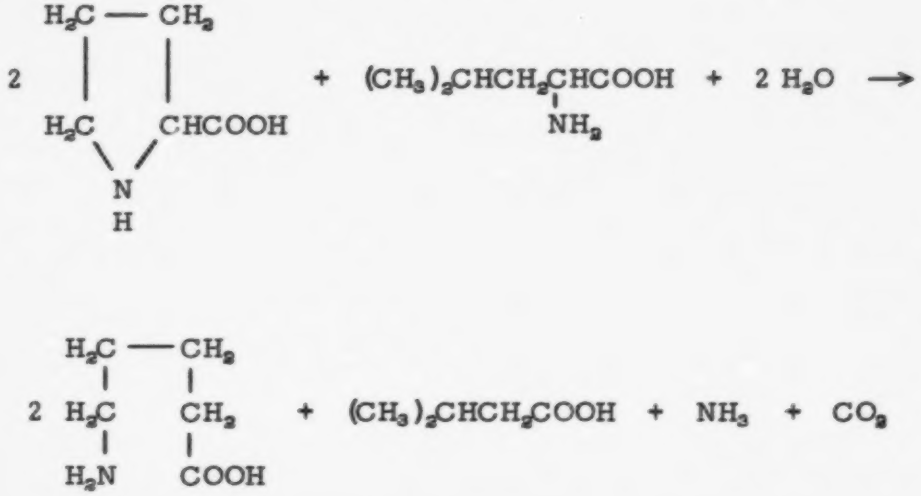
isovaleric acid



L-proline

δ-aminovaleric acid

Sum:



has been shown to serve as a source of carbon for fatty acid biosynthesis by *Lactobacillus arabinosus* and *Escherichia coli* (113).

Various species of *Pseudomonas* and *Escherichia* are found in anaerobic wastewater sludge; hence, methionine fermentations should occur.

Tyrosine Fermentations

A survey of the literature (through 1964) failed to indicate that tyrosine is fermented. Tyrosine can be deaminated and decarboxylated and can participate in transamination reactions by some microorganisms; however, there is no evidence that tyrosine is fermentable. The bacterial deamination of tyrosine results in the production of phenol, pyruvate, and ammonia (17,18,152). Tyrosine is decarboxylated by microorganisms to yield tyramine (104).

Tyrosine also is involved in an α-ketoglutarate-tyrosine transamination yielding glutamate as one of the products (44,102). *Pseudomonas fluorescens*, *Escherichia coli*, and *Leuconostoc arabinosus* were the organisms responsible for the transamination reaction. Various species of *Pseudomonas* and *Escherichia* are found in anaerobic wastewater sludge; hence, tyrosine fermentations should occur.

Summary

There seems to be no definitive data indicating the enzymatic decarboxylation of the following free amino acids to amines: glycine, alanine, serine, threonine, asparagine, glutamine, cysteine, cystine, methionine, proline, and hydroxyproline (104). If carbon dioxide is formed in the anaerobic decomposition of these amino acids, the products formed are not amines but are fatty acids, carbon dioxide, and ammonia. However, direct evidence is available for the fermentation of glycine, alanine, and methionine.

Leucine serves as the hydrogen donor in the anaerobic Strickland reaction. Leucine is also involved in transamination reactions catalyzed by organisms normally found in anaerobic wastewater sludge.

Tyrosine is also involved in transamination reactions with these same organisms.

3-7 UPTAKE STUDIES OF AMINO ACIDS BY WASTEWATER SLUDGES

In almost all of the reported uptake studies of amino acids by wastewater sludges, the amino acid was the sole nitrogen source or provided a significant part of the energy, carbon, or nitrogen. The lone exception is the glycine uptake studies by Woods (164) with anaerobic wastewater sludge. In this study (164) glycine was added to the wastewater sludge so that the concentration of the amino acid added was about 0.4 mg/l (5.3 micromoles/liter). This concentration is less than one percent of the concentration of amino acids normally found in wastewater sludge and is an insignificant nitrogen, carbon, and energy source. The results of this study (164) indicated that glycine was utilized by the wastewater sludge and the uptake rate of glycine was dependent upon the overall rate of metabolism taking place during the digestion process.

Carlson (28) studied the utilization of arginine, cystine, and glycine by activated sludge in which the amino acid was the sole nitrogen source and glucose was the carbon and energy source. The results of this study (28) indicated that activated sludges could become acclimated to a given amino acid as the sole nitrogen source and that sludges acclimated to a specific amino acid utilized this amino acid several times as fast as unacclimated sludges. The results of other studies (107,148) showed that amino acids were utilized in metabolic processes, but the uptake of amino acids was not evaluated.

Wojek (162) studied the decomposition of L-alanine during the anaerobic decomposition of synthetic sludge. Both the synthetic sludge and the L-alanine provided a nitrogen source for the microorganisms, but the alanine provided a large proportion of the nitrogen.

Only a few species of bacteria are known to derive energy from the fermentation of a single amino acid or other nitrogenous compounds (25,26). Clostridium propionicum ferments alanine and Diplococcus glycinophilus ferments glycine (26). The end products are fatty acids, ammonia, and carbon dioxide.

Amino acid uptake can be measured under two extreme conditions. The first condition is that in which the amino acid serves as the sole carbon source, or the sole energy source, or the sole nitrogen source, or any combination of these factors. Aerobic experiments with pure cultures of Escherichia coli showed that the uptake rate of amino acids was lower when the amino acid was the sole nitrogen and energy source than when the amino acid was the nitrogen source and glucose the energy source (21).

Other experiments by Roberts (120) with Escherichia coli under aerobic conditions showed that a period of acclimation of several days was required before D-glutamic acid and L-proline were utilized as the sole nitrogen source. However, acclimation was not necessary for L-glutamic acid, methionine, valine, glycine, and threonine to be utilized, although a reduced growth rate was observed for the first half hour (120).

All of the preceding studies and the conditions of the environment are different from those which exist in anaerobic digesters.

A second condition under which amino acid uptake can be evaluated is that in which other materials serve as the primary carbon source, nitrogen source, and energy source. The amino acid is added to this environment so that the concentration is less than 0.01 of the average total amino acid concentration or about 10 micromoles per liter (164). This concentration will not cause a great imbalance in the existing metabolic conditions. This method should measure the uptake rate potential of an amino acid that actually exists in the anaerobic wastewater sludge under prevailing conditions.

An aerobic culture of Escherichia coli grown in a medium in which glucose was the energy source and ammonia was the nitrogen source utilized glycine at concentrations as low as 0.1 micromoles/liter (80). The glycine uptake rate was essentially constant in the range of 10 to 100 micromoles/liter. The uptake rate slowly increased as the glycine concentration was increased from 100 to 100,000 micromoles/liter (80). *Escherichia coli* grown under aerobic conditions in a glucose medium will take up the glycine into the cell forming a glycine pool, and then within a few minutes the glycine leaves the pool and is utilized in the formation of proteins (21,92).

3-8 COMMENTS ON THE LITERATURE

The digestion of wastewater sludge and the biochemistry of amino acids in an anaerobic environment are very complex. Wastewater sludges may contain relatively constant amounts of total organic material including proteins, carbohydrates, and lipids; but the various organic compounds in these groups are not constant. The heterogeneous character of the sludge does not provide an ideal environment for the microbial population. Slight disturbances can easily upset any balance that might have existed. A number of species of organisms are responsible for the enzyme-catalyzed hydrolytic reactions prior to the fermentation reactions. The waste organic materials, as well as the intermediate end products of the metabolic activity of the organisms, may result in substances that are antagonistic to the microbial population.

The studies cited in the literature were for the most part carried out with pure cultures and defined substrates, and even under these conditions the environment is very complex. The study of a heterogeneous culture, such as exists in an anaerobic sludge digestion unit, is a very complex undertaking.

CHAPTER 4

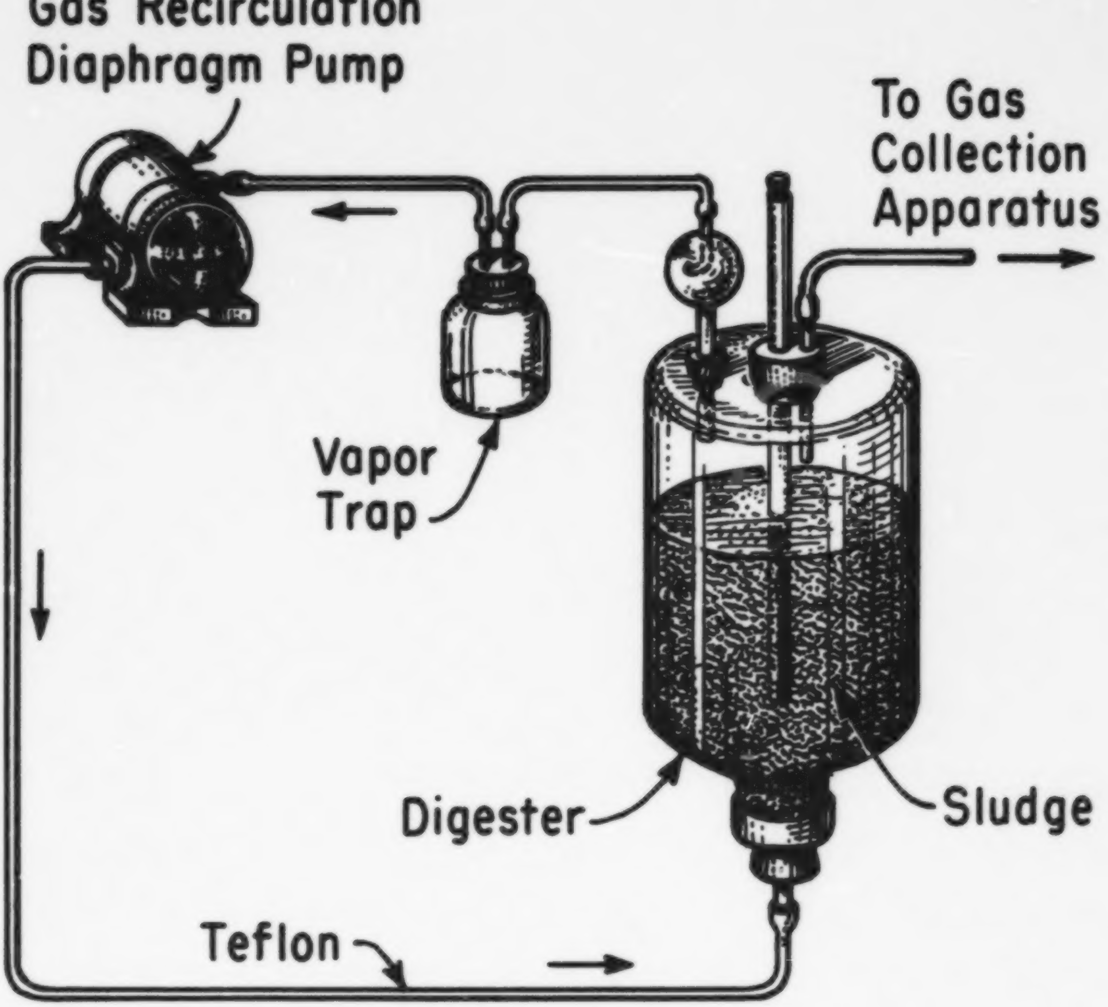
EQUIPMENT AND EXPERIMENTAL PROCEDURES

The primary investigation consisted of uptake and utilization studies on amino acids during the anaerobic digestion of wastewater sludge. The inoculum for these studies was obtained from laboratory bench-scale digestion units. A carbon balance of this inoculum was necessary for the proper evaluation of the amino acid uptake and utilization. The extent of destruction of the proteins, carbohydrates, lipids, free amino acids, volatile solids, and total solids was determined.

4-1 BENCH-SCALE DIGESTERS

The bench-scale digesters were operated on a fifteen-day detention period and were fed primary sludge once daily. The digestion apparatus is shown in Fig. 4-1. A 10-liter bottle was used as the digester. During normal operation, the volume of sludge was maintained at 6 liters. The volume of sludge removed was equal to the volume of feed material added daily, namely 400 ml. A diaphragm pump was used to recirculate the gases produced by digestion for fifteen minutes out of each hour. The gases were collected over saturated brine in a 20-liter bottle. The four digesters were maintained at 35°C by use of a water bath.

Samples and feed material were withdrawn and added through the same tube. A vacuum pump with a trap was used to withdraw the sample from the digester. Prior to the withdrawal of a sample, gas was


Gas Recirculation
Diaphragm Pump
To Gas
Collection
Apparatus
Vapor
Trap
Digester
Sludge
Teflon

LABORATORY SCALE DIGESTER
(THE UNIVERSITY OF TEXAS)
FIG. 4-1

recirculated for fifteen minutes and the digester was manually shaken. As the sample was withdrawn, the displaced volume was occupied by the gases that had been collected in the brine bottle. Upon the addition of the feed material, the gases were forced back into the gas collection system. The only air added to the digester was that air trapped in the sludge since the sampling tube extended below the level of the sludge in the digester.

4-2 EXPERIMENTAL PROCEDURES

The experiments were designed for (1) determining the uptake rate of the amino acid, (2) differentiating between the biological and non-biological uptake of the amino acid, (3) measuring the quantity of carbon dioxide and methane gas produced from the amino acid, (4) evaluating the total gas production rate from the sludge and the amino acid and the effect of the addition of an amino acid on this rate, and (5) determining the intermediate breakdown products of the amino acid if present in sufficient quantity.

The uptake rate was determined by adding 5 microcuries (μc) of a C-1-14 amino acid being studied to 15 ml of the digesting sludge in Warburg flasks (10 ml of sludge for the glycine and tyrosine experiments). The weight added varied for each amino acid, but the activity was held constant (glycine was an exception, 1 μc glycine was added). The uptakes after 5, 10, 30 minutes, 1, 3, 6, 12, 24, 48, and 96 hours were determined. In the 35°C uptake studies (e.g., biological plus non-biological) the sludge was added to helium-flushed Warburg flasks which were then flushed with digester gas. The tagged amino acid was added to these flasks through serum caps. At the proper time the biological uptake was stopped by the addition of 25 percent trichloroacetic acid so that the final concentration of trichloroacetic acid was 5 percent and the pH about 2. The gases produced in the Warburg flasks were withdrawn with a 10-ml gas-tight syringe, separated by gas chromatography, and injected into an evacuated Nuclear Chicago ionization chamber for subsequent measurement. The remaining gas in the Warburg flasks was displaced by helium and exhausted through a hood. Within 10 minutes after stopping the biological uptake, the wastewater sludge was centrifuged and the supernatant retained for measurements of its activity. In order to measure the activity in the supernatant, a 10-microliter volume of the supernatant was added to a 54-millimeter diameter Whatman Number 1 filter paper in such a manner as to cover a circle with a 1/2 inch diameter and evaporated. A quantitative measure was made possible by comparing the data with a standard solution prepared from the supernatant of the sludge being tested, adjusted to pH 2, to which the tagged amino acid is added.

In order to verify that the activity in the supernatant was associated with the applied tagged amino acid and not with one of its decomposition products, a paper chromatogram was performed with subsequent exposure of the chromatogram to X-ray film for periods of more than three to four weeks. The radioautographs served the purpose of locating the activity (amino acids or intermediate breakdown products) on the paper chromatograms prior to its measurement.

In order to differentiate between biological and non-biological uptake, an uptake experiment was performed at 4°C. It is assumed that no biological activity occurred at this temperature.

The measurement of the activity of the liquid-tagged amino acid samples applied to filter paper was done with a Sharp's Lowbeta Counter. This counter is described in Appendix C-1. The measurements of the activity of the carbon-14 tagged gas samples was done with a Nuclear Chicago Dynacon 6000 Electrometer. This instrument is described in Appendix C-4. The separations of the radioactive carbon dioxide and methane gas were done with a Beckman GC-2A Gas Chromatograph. This instrument is described in Appendix C-3.

The total uptake and utilization studies were done in specifically designed 125-ml flasks in a Precision Warburg Apparatus. Techniques for these studies are described in Appendix B-8.

CHAPTER 5

LABORATORY ANALYSES

A 400-ml sample of sludge was required for a complete analysis. The analysis of a sludge sample included pH and the concentrations of alkalinity, volatile acids, total solids, volatile solids, ammonia nitrogen, organic nitrogen, carbohydrates, proteins, lipids, free amino acids, and total carbon.

5-1 SAMPLE PREPARATION

The samples of feed material or digester effluent were placed in Erlenmeyer flasks and stored in a refrigerator until analysis. The analysis of the samples was usually completed within one week after the samples were collected.

Sample preparation generally consisted of dividing the samples into two categories. The first portion was the sludge as it was sampled and contained both liquid and solids. The second fraction was the decantate collected after a portion of the sludge was centrifuged at 21,000 gravities for 30 minutes. Most of the tests performed required that the samples be diluted to various strengths with ammonia-free distilled water. The details for sample preparation are given in Appendix B-1.

5-2 TOTAL SOLIDS AND VOLATILE SOLIDS

The contents of total solids and volatile solids were estimated by placing a known volume of the sludge in an evaporating dish. Total solids were those solids remaining after the water was driven off by heating at 103°C overnight. The volatile solids were those that were driven off when the solids were heated at 600°C for one hour.

5-3 pH, ALKALINITY, AND VOLATILE ACIDS

pH was determined by use of a pH meter (Beckman Model N), with a glass electrode and a calomel electrode. The alkalinity was estimated by titration with sulfuric acid. The volatile acids concentration was determined by direct titration as described by DiLallo and Albertson (38). (See Appendix B-2.)

5-4 AMMONIA NITROGEN AND ORGANIC NITROGEN

Ammonia nitrogen and organic nitrogen were determined as outlined in Standard Methods (9).

5-5 CARBOHYDRATE DETERMINATION

The concentration of carbohydrates was measured by making use of the colorimetric anthrone reaction. When anthrone is mixed with concentrated sulfuric acid and a carbohydrate, a blue or green colored complex is formed. The intensity of color is proportional to the concentration of carbohydrate.

The technique for carbohydrate analysis is a modified version of that described by Gaudy (59). (See Appendix B-3).

5-6 PROTEIN DETERMINATION

The proteins concentration was estimated by the Folin Reaction (85), a colorimetric test that depends upon the formation of a blue color which results from a reaction between the aromatic portions of the protein (or amino acid) and the reagents. (See Appendix B-4).

5-7 LIPID DETERMINATION

Lipids were extracted with a wet extraction method utilizing chloroform and methanol (84). The amount of lipids was determined gravimetrically. (See Appendix B-5).

5-8 FREE AMINO ACIDS DETERMINATION

The concentration of free amino acids was estimated by a colorimetric ninhydrin reaction (115,125). This reaction is not specific for amino acids; therefore, interfering compounds such as proteins and ammonia were removed prior to analysis. No correction was made for possible peptide interferences. (See Appendix B-6).

5-9 TOTAL CARBON DETERMINATION

Total carbon was determined by a wet oxidation method (109,155,156) in which the organic carbon and inorganic carbon are oxidized to carbon dioxide which is trapped in a standard sodium hydroxide solution. After proper manipulation, the excess sodium hydroxide is determined by titration. A quantitative measure of the amount of carbon in the sample is thereby obtained. (See Appendix B-7).

CHAPTER 6

EXPERIMENTAL RESULTS

Anaerobic fermentation of organic material in wastewater sludge is a complex biological and biochemical process. The mechanism by which carbonaceous and nitrogenous compounds are stabilized during anaerobic digestion of wastewater sludges are not established in sufficient detail. The objective of this study was to trace the anaerobic biological breakdown and utilization of individual amino acids added to wastewater sludge. Five amino acids, namely, glycine, alanine, leucine, methionine, and tyrosine, were studied individually in this anaerobic environment.

Three types of experiments involving amino acids were performed and include:

1) The effect of a single non-radioactive amino acid on gas production,

2) The degradation of a non-radioactive amino acid, and

3) Radioactive amino acid uptake and utilization.

In the first experiment the effect of glycine on the overall anaerobic metabolism of the wastewater sludge was studied and was measured in terms of the total gas produced at initial concentrations of glycine in a 15-ml sludge volume of 0, 1.08, and 4 millimoles per liter. The total quantity of gas produced was measured utilizing Warburg techniques. These data are presented in Fig. 6-1 and Table A-1 in the Appendix. The components of the gas, carbon dioxide and methane, were qualitatively and quantitatively analyzed by gas chromatography.

In the second series of experiments, the deaminative degradation of individual non-radioactive amino acids (i.e., glycine, alanine, methionine, and tyrosine) during anaerobic sludge digestion was evaluated by means of a nitrogen balance before and after the addition of the amino acid under consideration. This balance included the redistribution of ammonia nitrogen and organic nitrogen in the sludge supernatant and unaltered sludge. These data are tabulated in Table A-3 in the Appendix.

In the third series of experiments, the uptake and utilization of a single 1-C-14 labeled amino acid (i.e., glycine, L-alanine, L-leucine, DL-methionine, and L-tyrosine) by anaerobic wastewater sludge was evaluated in Warburg flasks. This study included a radioactive materials balance after various time intervals and measurements of the gas production rates with a Warburg apparatus. The results of the materials balance are presented in Tables A-5 through A-14, and the gas production curves are shown in Figs. 6-2 through 6-6.

6-1 WASTEWATER SLUDGE DIGESTION

The uptake and utilization of amino acids by wastewater sludge in an anaerobic environment was evaluated. Wastewater sludge is a variable, undefined substrate which contains a heterogeneous mixture of microorganisms. Therefore, the characteristics of the sludge and the effects of the amino acid on this sludge are necessary for the proper evaluation of uptake data.

The physical and chemical characteristics of the substrate are summarized in Table A-2 in the Appendix. Only the characteristics of the sludge samples which were used as the substrate in the radioactive uptake and other studies were recorded. The data indicate that the laboratory-scale digestion units were operating at conditions close to steady-state. The data describing the characteristics of the sludge allow the evaluation of the effects of the addition of an amino acid on the environment within the digester.

The analysis of sludge by the techniques described in Appendix B yields approximate results because of the heterogeneity of the sludge mass in terms of particle size, distribution of particles, and organic composition of the sludge. This factor of non-homogeneity and discrete particles is responsible to a great extent for the variability in the data recorded for the various groups of organic compounds. This variability was most evident in the attempts to obtain a nitrogen and carbon balance which was only partially successful. However, these data in conjunction with the radioactive studies can supply useful information concerning the breakdown and utilization of amino acids which were added to anaerobically digesting wastewater sludge.

The characteristics of the sludge samples are summarized in Table A-2. These data were used to evaluate the effects of the amino acid on the degradation of digesting wastewater sludge.

pH

The data indicate that the pH of the sludge for almost all amino acid experiments was 8.0. The addition of amino acid to the sludge in concentrations of up to 5 mM/l did not change the pH.

Alkalinity

Most of the alkalinity in the digesting sludge resulted from the recombination of the products of liquefaction and gasification in the form of carbonates and bicarbonates. The addition of amino acids to the sludge resulted in an increase in the alkalinity which may have been caused by the recombination of the carbon dioxide produced during the fermentation of the amino acids and other intermediate products. A concentration of 5 mM/l of an amino acid resulted in an average increase in alkalinity of about 200 mg/l as calcium carbonate. The range of alkalinity was about 3,000 to 3,200 mg/l as $CaCO_3$.

Volatile Acids

The volatile acids (i.e., short chain fatty acids) are intermediate products of liquefaction of volatile solids and serve as the substrate for the gasifying organisms. The data indicate that the volatile acid concentration increased after the addition of an amino acid to the sludge. The volatile acid concentration was maintained in the range of about 100 to 700 mg/l as acetic acid. The average increase in the volatile acid concentration resulting from the addition of 5 mM/l of amino acid was about 100 mg/l as acetic acid.

Volatile Solids

The data indicate that operation under a 15-day detention period and an organic loading of about 0.2 pounds of volatile solids per cubic foot per day resulted in a reduction in the concentration of volatile solids from about 32,000 mg/l to 22,000 mg/l. This decrease in the volatile solids concentration indicates an active environment and sufficient available substrate for the amino acid experiments.

Carbohydrates

Carbohydrates are a primary energy source for most microbial processes. The data indicate a reduction in carbohydrate concentration from about 10,000 to 3,500 mg/l reported as glucose.

Lipids

The lipids serve as an energy source for both the acid-producing and the methane-forming bacteria. Fatty acids are intermediate products of lipid degradation and these fatty acids serve as the carbon and energy source for some methane bacteria (16). A reduction in lipid concentration from about 7,000 to 3,000 mg/l was recorded.

Proteins

The protein concentration decreased from about 12,500 to 9,000 mg/l as bovine serum albumin. The hydrolysis of proteins to amino acids accounts for this reduction in protein concentration.

Free Amino Acids

The concentration of free amino acids in the wastewater sludge used as feed for the laboratory digester was about 700 mg/l reported as glycine; however, the free amino acid concentration in the sludge withdrawn from the laboratory digesters generally was greater than 800 mg/l reported as glycine.

The addition of 1 mM/l of non-radioactive glycine to the sludge resulted in utilization of the amino acids and in stimulation of gas production during the Warburg studies. The data are summarized in Table A-1 and graphically in Fig. 6-1. The data indicate that a concentration of 4 mM/l of non-radioactive glycine was inhibitory and resulted in a decrease in the gas production rate from 10 to 6 liters per day in the laboratory digesters. The results of other studies in which 5 mM/l of non-radioactive amino acids were added to the laboratory digesters indicate that at this concentration methionine, tyrosine, and leucine caused a drop in the gas production.

The non-radioactive amino acid uptake data indicate that concentrations of 4 mM/l or greater are inhibitory to the overall anaerobic sludge digestion process. The data also indicate that at a concentration of up to 1 mM/l the amino acid stimulates gas production. This stimulatory effect was also observed during the radioactive amino acid studies with all five of the amino acids. Stimulation is defined as an increase in gas production over that amount of gas which would result directly from the decomposition of the amino acid.

Organic Nitrogen and Ammonia Nitrogen

The organic nitrogen in sludges is mainly in the form of proteins and amino acids. The products of hydrolysis of the proteins are amino acids which are utilized by the microorganisms present in the anaerobic environment and converted to ammonia nitrogen.

The data indicate that during the course of digestion the ammonia nitrogen concentration increased from about 300 mg/l to about 800 mg/l and the organic nitrogen content decreased from about 1,500 mg/l to 1,200 mg/l. The data also indicated a decrease in protein concentration and an increase in free amino acid concentration during digestion. This conversion of proteins to amino acids does not change the organic nitrogen content. However, the degradation of the amino acids results in an increase in ammonia nitrogen and a decrease in organic nitrogen. The conversion of organic nitrogen in the form of an amino acid to ammonia nitrogen is illustrated by the data in Table A-3.

Nitrogen Balance

A nitrogen balance was evaluated in conjunction with each amino acid uptake study. This balance includes the change in organic and ammonia nitrogen resulting from the addition of an amino acid. These nitrogen balances will be discussed in section 6-3 of this chapter.

Total Carbon

The total carbon content of the digesting sludge decreased as the stabilization of the volatile solids progressed. The organic carbon was biologically converted to methane and carbon dioxide gas. The data indicate a reduction in concentration of total carbon from about 19,000 mg/l to 13,000 mg/l.

Carbon Balance

A carbon balance was performed in conjunction with each amino acid uptake experiment. These balances are presented in Table A-4 in the Appendix. The carbon balance equation may be written as follows:

$$\text{Total carbon in feed} = \text{Total carbon in digester} + \text{Total carbon in gas produced}$$

The gas contains about 65 percent methane and 35 percent carbon dioxide, and a liter of gas contains 537.4 mg of carbon at standard temperature and pressure (164).

Gas Production

The volume of gas was about 1 liter of gas per day per liter of sludge and varied with the volatile solids concentration in the feed sludge. The addition of 5 mM/l of amino acids to the laboratory-scale digesters lowered the rate of gas production. This condition also existed in completely mixed Warburg studies with glycine at 4 mM/l (Table A-1). The composition of gas in the laboratory digesters was 65 percent methane, 34 percent carbon dioxide, and 1 percent nitrogen or other gases.

It should be noted that a concentration of 5 mM/l of an amino acid caused no measureable pH change, an increase in volatile acids concentration of about 100 mg/l and in alkalinity of about 200 mg/l. A drop in gas production from 7 to 4 liters/day with no recovery in one week has been observed for methionine. The addition of tyrosine caused a continuous drop from 7.1 to 1.3 liters/day over a seven-day period. The gas production decreased from 4.3 to 3.5 liters/day immediately after the addition of leucine and increased to 5.0 liters/day within four days. Glycine caused a drop in gas production from about 10.0 to 6.4 liters/day over a period of four days after which the gas production rate at this lower level stabilized. Inconclusive results were obtained after adding alanine. These data show that high concentrations of amino acids can have inhibitory effects on the overall metabolic activity of the digester.

6-2 NON-RADIOACTIVE STUDIES

Gas production was used as a measure of the effectiveness of the anaerobic digestion process on the stabilization of wastewater sludge. The rate of gas production from small volumes of sludge can be measured with the Warburg technique. A change of the substrate may result in a minute change in the gas production rate which may be detectable with the Warburg apparatus. Wastewater sludge contains a multitude of organic compounds which may be biologically degraded to gaseous end products, primarily carbon dioxide and methane. The addition of a single amino acid to this anaerobic environment may affect the rate of gas production depending on concentration added. At very low amino acid concentrations, the gas produced from the decomposition of the sludge components would exceed any gas contributed by the amino acid. At moderate amino acid concentrations, the degradation of some of the amino acid to gaseous products may result in an increase in the rate of gas production over that observed for the sludge alone. High amino acid concentrations may have an inhibitory effect on the system and the gas production rate could decrease.

A measure of the rates of gas production after the addition of an amino acid to wastewater sludge should provide supplementary information regarding the degradation and utilization of amino acids in the anaerobic environment.

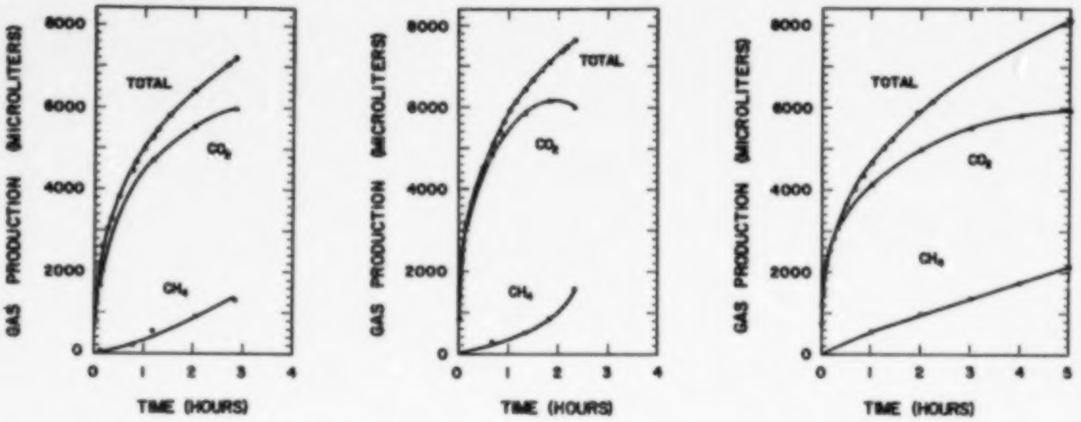
The anaerobic decomposition of wastewater sludge is a complex biological system in which a large number of variables exist and may result in inconsistent and sometimes unreliable data when non-radioactive amino acids are used. However, radioactive studies in the same environment utilize experimental techniques that result in more accurate data and lead to more reliable interpretations of the results.

The data observed when non-radioactive glycine was added to the sludge indicate that during the anaerobic decomposition of wastewater sludge 300 µl of gas was produced for each ml of sludge (0.79 µl per mg of volatile solids added or 1.31 µl per mg of total solids added) during the first hour in the Warburg flasks. The effect of glycine on gas production was evaluated by using glycine at a concentration of 1.08 mM per liter and 4.0 mM per liter, respectively.

The data indicate that the addition of 1.08 mM per liter of glycine stimulated gas production immediately. However, at a concentration of 4.0 mM per liter, glycine was initially inhibitory, but the system became acclimated after about two hours. The gas production data are shown in Table A-6. Figure 6-1 is a graphical illustration of the time variation of total gas production, carbon dioxide production, and methane production for each concentration of glycine. The carbon dioxide and methane content of the gas are theoretical curves based on the chromatographic analyses of gas samples.

A maximum increase in the amount of gas produced after the addition of 1.08 mM/l of glycine for two separate runs was 400 µl and 950 µl. Based on the equation of Cardon (27), the theoretical amount of gas produced should have been about 380 µl. Additional discussion of this concept is presented in section 7-2. The Warburg technique is a reliable means of measuring changes in gas volume, and the discrepancy in the maximum increase in gas production may be attributable to the variability and heterogeneity of the sludge samples. In addition, it is possible that the amino acid provided a stimulatory effect and increased the overall degradation rate. This stimulatory effect is discussed in more detail in Chapter 7.

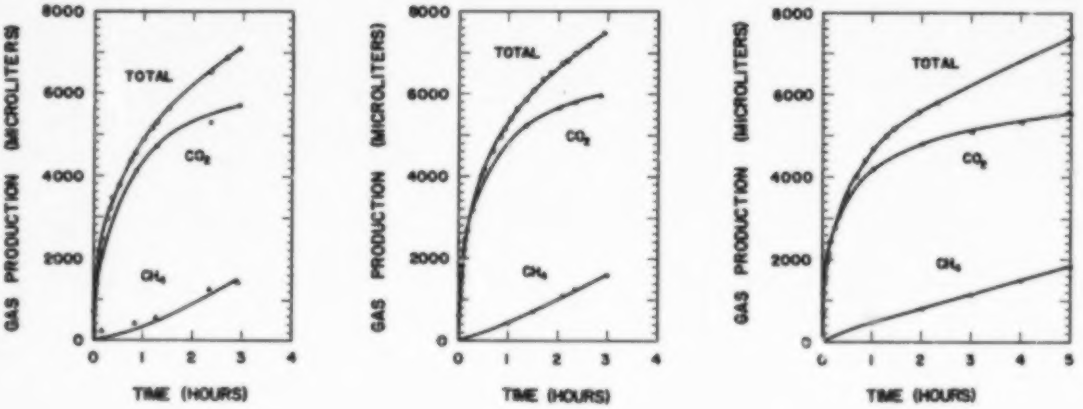
The increased quantity of gas resulted from the production of carbon dioxide during the anaerobic degradation of glycine (27) and the subsequent reduction or recombination of a portion of this carbon dioxide to methane gas. Although carbon dioxide is formed, these data do not



RUN 1
NO GLYCINE ADDED

RUN 1
1.08 $\frac{mM}{L}$ GLYCINE

RUN 1
4.0 $\frac{mM}{L}$ GLYCINE



RUN 2
NO GLYCINE ADDED

RUN 2
1.08 $\frac{mM}{L}$ GLYCINE

RUN 2
4.0 $\frac{mM}{L}$ GLYCINE

FIG. 6-1 THE QUANTITATIVE AND QUALITATIVE EFFECTS OF GLYCINE CONCENTRATION ON GAS PRODUCTION DURING ANAEROBIC DIGESTION

indicate decarboxylation since the intermediate product in the anaerobic degradation of glycine is acetic acid (15,27,41,124,135,136,137) and no data which indicate the enzymatic decarboxylation of glycine by bacteria to amines have been reported (104).

6-3 NITROGEN BALANCE AFTER ADDING AMINO ACIDS

An amino acid which is added to wastewater sludge in an anaerobic environment is either incorporated into protein or degraded to some other compounds. The amino acid is originally in the form of organic nitrogen. An increase in ammonia nitrogen content of the sludge indicates that ammonia has been released, i.e., deamination takes place. Concentrations of glycine of 1,000, 2,000, and 3,000 mg/l (187, 374 and 560 mg nitrogen/liter, respectively) were used since the Kjeldahl technique is a relatively insensitive and inaccurate procedure to indicate the trend in nitrogen distribution at lower concentrations of amino acids in sludge. Concentrations of about 70 mg of nitrogen per liter were used in the alanine, methionine, and tyrosine studies. These data are summarized in Table 6-1. However, inconclusive results were obtained with leucine; therefore, data are not presented. High amino acid concentrations were used in order to make the amino acid the dominant nitrogen source and to be able to detect any changes adequately by the Kjeldahl technique. However, concentrations as high as 3,000 mg of glycine per liter may have an inhibitory effect. The analyses of total free amino acids in the sludge withdrawn from the laboratory-scale anaerobic digester were in the

Summary Table 6-1

Nitrogen Balance

Amino Acid	Amount of Amino Acid Added mg/l	Amount of Nitrogen Added mg/l	Increase in[(a)] NH_3-N in Total Sample mg/l
Glycine	3000	560	92
Glycine	3000	560	313
Glycine	3000	560	134
Glycine	3000	560	92
Glycine	2000	374	84
Glycine	1000	187	105
Alanine	445	70	312
Methionine	747	70	127
Tyrosine	906	68	-22

(a) See data in Table A-3 in the Appendix.

range of 800 mg per liter. Therefore, the concentration of a single amino acid to be added was below 1,000 mg per liter so that the anaerobic environment was not seriously disturbed.

The data indicate that for glycine, alanine, and methionine there is a significant decrease in organic nitrogen and an increase in ammonia nitrogen. The data for tyrosine and leucine were inconclusive.

On the basis of the data obtained it is evident that deamination is a major reaction in the anaerobic degradation of an amino acid.

6-4 GAS PRODUCTION DURING AMINO ACID 1-C-14 STUDIES

The uptake and utilization of radioactive amino acids were evaluated using Warburg techniques; therefore, total gas production rates and uptake of C^{14} could be followed simultaneously (See Appendix B-9). This study provided a method of observing the effect of amino acids on the environment and a measure of the total gas production rate before and after the addition of the amino acid. The gas produced included radioactive and non-radioactive carbon dioxide and methane gas.

A comparison of the actual gas produced after the addition of the amino acid and the theoretical amount that should be formed from the degradation of the amino acid can be evaluated. The radioactive carbon dioxide and methane gas produced indicate the actual gas resulting from the 1-C-14 amino acid. Any gas produced in excess of that amount produced during digestion prior to the addition of the labeled amino acid indicates that the amino acid stimulated the overall metabolism of the environment.

The data indicated that after the addition of a small amount of a single amino acid, a measureable increase in the amount of gas produced was observed. The amount of each amino acid added was 12.0 μM/l of glycine, 42.1 μM/l of L-alanine, 13.2 μM/l of L-leucine, 104.5 μM/l of DL-methionine, and 18.8 μM/l of L-tyrosine. Each amino acid was studied for a period of 96 hours. Typical rates of gas production prior to the addition of the amino acid and after the addition of the amino acid are shown in Fig. 6-2 through 6-6. Ten separate flasks were used in the radioactive uptake studies.

A measureable change in the rate of gas produced was observed for these amino acids. Gas production data were collected during the contact periods with the radioactive amino acids. The variations in the gas production rates may be attributable to the environmental conditions since the sludge and microbial flora are not precisely the same even though the inoculum was taken from the same source. Complete data or graphs on all ten studies are not presented since the variability of data can easily be shown with about three graphs for each of the five amino acids studies (Fig. 6-2 through 6-6).

The data presented in Table 6-2 illustrate (1) the gas production rate from the sludge, (2) the gas production rate after an amino acid is added to the sludge, and (3) the thecretical amount converted to gas from the amino acid 1-C-14 added. These data indicate that the amount of radioactive gas produced from the amino acid ranged from a minimum value of 1.12 μl/hr for glycine to a maximum of 4.82 μl/hr for alanine.

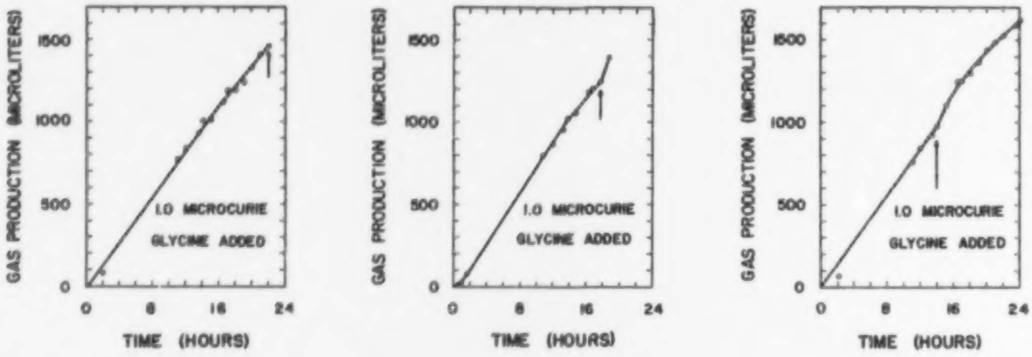


FIG. 6-2 STIMULATORY EFFECTS OF GLYCINE 1-C-14 ON GAS PRODUCTION

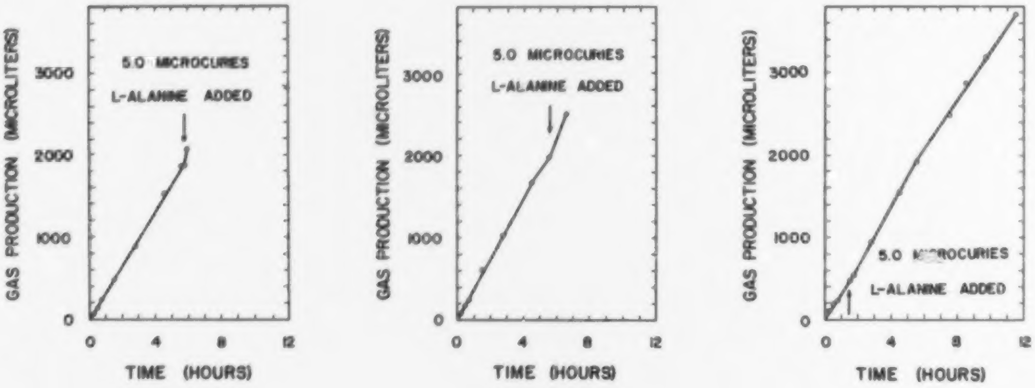


FIG. 6-3 STIMULATORY EFFECTS OF L-ALANINE 1-C-14 ON GAS PRODUCTION

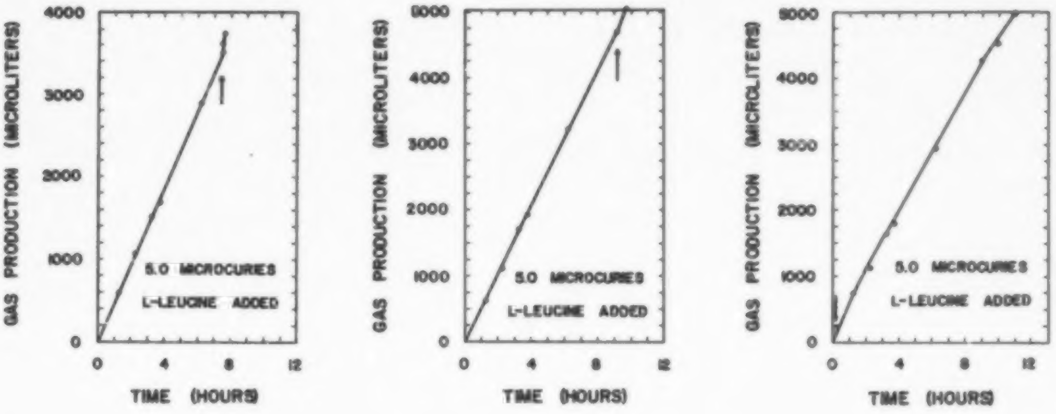


FIG. 6-4 STIMULATORY EFFECTS OF L-LEUCINE I-C-14 ON GAS PRODUCTION

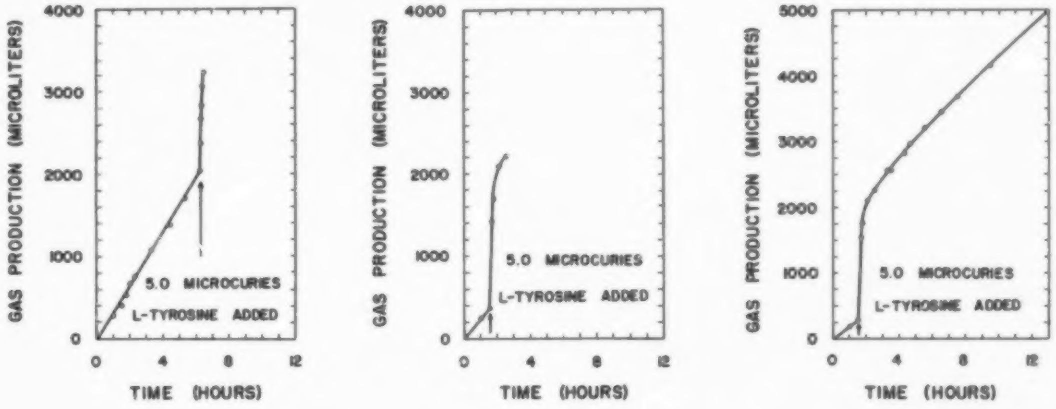


FIG. 6-5 STIMULATORY EFFECTS OF L-TYROSINE I-C-14 ON GAS PRODUCTION

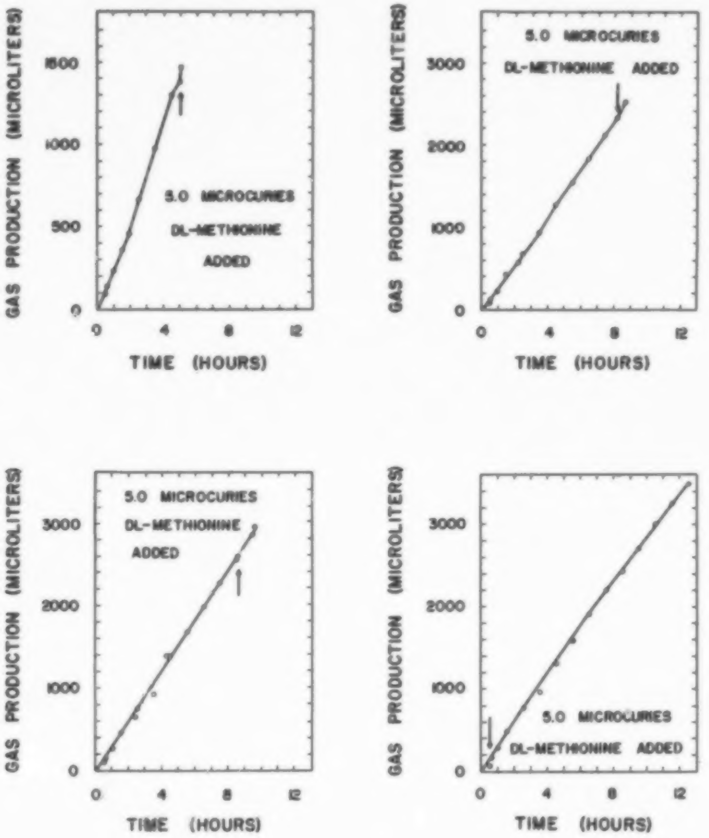


FIG. 6-6 STIMULATORY EFFECTS OF

DL-METHIONINE 1-C-14 ON GAS PRODUCTION

Table 6-2

Gas Production Rates

Amino Acid	Range of Maximum Gas Production Rates Before the Addition of the Amino Acid 1-C-14 as Measured Manometrically by the Warburg Apparatus	Range of Maximum Gas Production Rates After the Addition of the Amino Acid 1-C-14 as Measured Manometrically by the Warburg Apparatus		Maximum Gas Production Rate Computed from the Measured Activities of the $C^{14}O_2$ and $C^{14}H_4$ Gas Produced from the Amino Acid 1-C-14 in the Warburg Apparatus	
	μl/hr	μl/hr	See Fig. for Rate Curve	μl/hr	See Table
Glycine	68.5 - 78.3	111 - 146	6-2	1.12	A-6, A-15
L-alanine	313 - 371	367 - 1120	6-3	4.82	A-8, A-15
L-leucine	472 - 527	575 - 2650	6-4	1.79	A-10, A-15
DL-methionine	287 - 325	303 - 1150	6-6	2.94	A-12, A-15
L-tyrosine	190 - 326	5330 - 7020	6-5	2.52	A-14, A-15

However, the results of Warburg studies conducted simultaneously indicate an increase in total rate of gas produced in the range of 41 μl/hr for glycine to about 6,700 μl/hr for tyrosine. All the gas produced was measured by the Warburg technique. These results indicate that the presence of small amounts of the radioactive amino acids stimulated gas production during the anaerobic degradation of wastewater sludge.

6-5 RADIOACTIVE CARBON BALANCES

The uptake and utilization of amino acids during anaerobic digestion were evaluated based on experimental procedures consisting of (1) estimating the total uptake and the rate of uptake of a single amino acid 1-C-14, (2) differentiating between the uptake at a temperature of 35°C (biological) and at 4°C (non-biological), (3) qualitatively and quantitatively analyzing the gas produced, (4) estimating the amount of free amino acid remaining in the substrate, and (5) determining the presence of tagged decomposition products in the substrate other than the original tagged amino acid compound. Detailed procedures are presented in Appendix B-9.

The data are presented in terms of nanograms (ng) of amino acid per milligram of volatile solids (ng/mg VS). Gas production data are presented in terms of ng of amino acid converted to gas per mg VS to facilitate an amino acid materials balance.

The data are tabulated in Tables A-5 through A-17 in the Appendix. The graphical presentation of these data in Fig. 6-7 through 6-21 includes a series of three graphs for each amino acid studied. The amount of amino acid 1-C-14 added to the wastewater sludge and the distribution of the labeled amino acid in solution, in the gas, and associated with the sludge mass at different time intervals are illustrated in the first graph of the series. The second graph shows the conversion of amino acid 1-C-14 to radioactive carbon dioxide gas and radioactive methane gas. The uptake and utilization of the amino acid 1-C-14 is presented in two curves in the third graph. One curve represents the uptake and utilization of the labeled amino acid by the sludge mass and bacteria at 35°C (biological uptake). The second curve shows the uptake of the amino acid by the sludge mass at 4°C (non-biological uptake). The data from the amino acid 1-C-14 chromatograms are presented in Table A-18 in the Appendix. The characteristics of the sludge inoculum for these studies are presented in Table A-2 in the Appendix.

The data indicate that at the end of the 96 hour test period at 35°C 0.86 ng of glycine 1-C-14 per mg VS or 2.38 percent of the glycine 1-C-14 added remained in solution, 40.8 percent appeared as gas, and 56.8 percent was associated with the sludge solids. Within five minutes at 35°C the total uptake (by the sludge mass) of glycine 1-C-14 was 16.3 ng per mg VS or 45.2 percent of the total available glycine 1-C-14 of 36.1 ng per mg VS. After six hours the maximum uptake at 35°C was 31.8 ng per mg VS or 88.2 percent. The maximum uptake rate at 35°C occurred during the first five minutes and was 195.6 ng per mg VS per hour; however, the maximum uptake at 4°C (non-biological) was 33.5 ng per mg VS

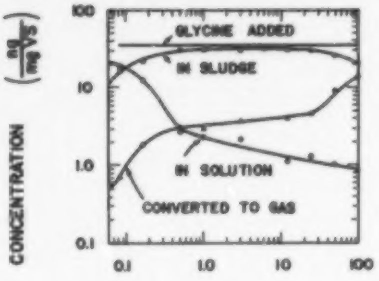


FIG. 6-7 DISTRIBUTION OF

GLYCINE 1-C-14 AT 35°C

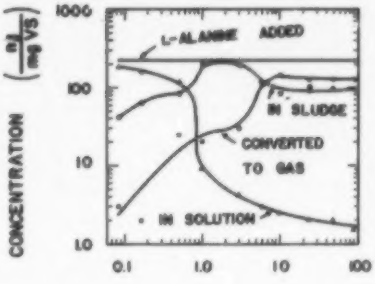


FIG. 6-10 DISTRIBUTION OF

L-ALANINE 1-C-14 AT 35°C

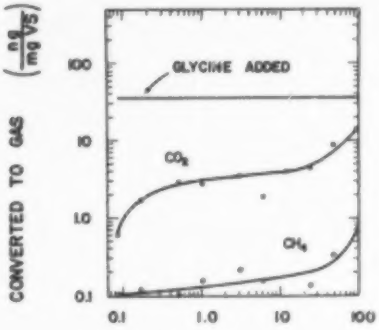


FIG. 6-8 CONVERSION OF GLYCINE

1-C-14 TO RADIOACTIVE GASES AT 35°C

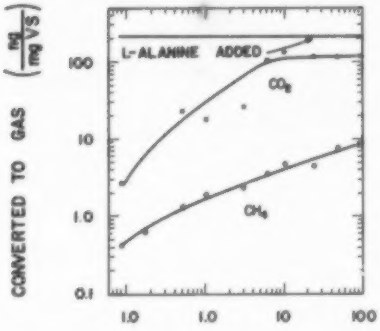


FIG. 6-11 CONVERSION OF L-ALANINE

1-C-14 TO RADIOACTIVE GASES AT 35°C

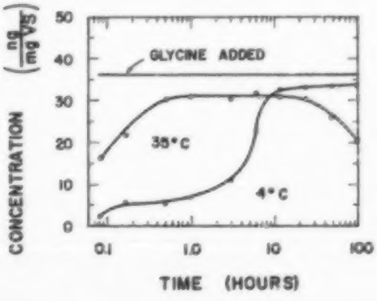


FIG. 6-9 UPTAKE AND UTILIZATION OF

GLYCINE 1-C-14 (4°C AND 35°C)

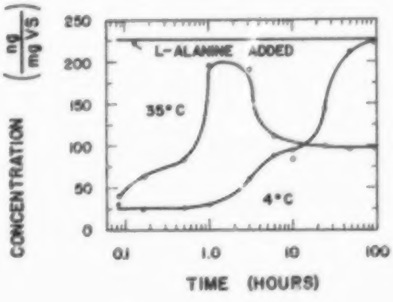


FIG. 6-12 UPTAKE AND UTILIZATION OF

L-ALANINE 1-C-14 (4°C AND 35°C)

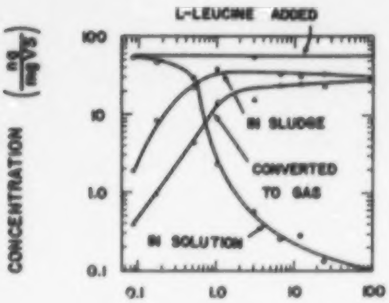


FIG. 6-13 DISTRIBUTION OF L-LEUCINE I-C-14 AT 35°C

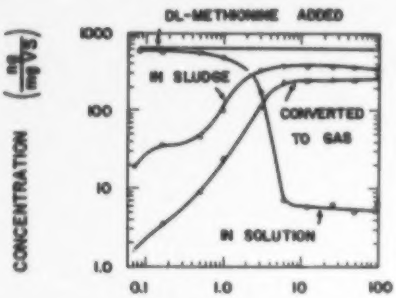


FIG. 6-16 DISTRIBUTION OF DL-METHIONINE I-C-14 AT 35°C

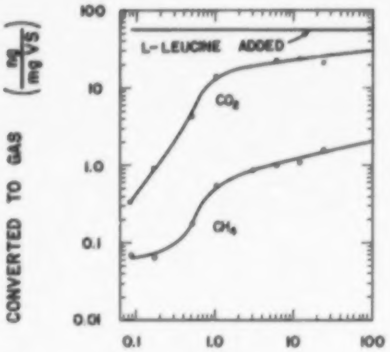


FIG. 6-14 CONVERSION OF L-LEUCINE I-C-14 TO RADIOACTIVE GASES AT 35°C

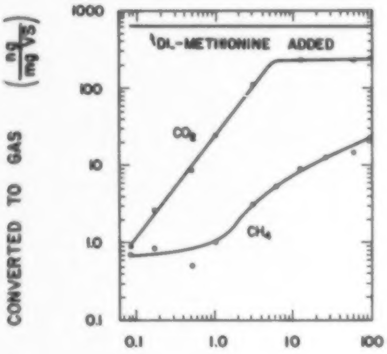


FIG. 6-17 CONVERSION OF DL-METHIONINE I-C-14 TO RADIOACTIVE GASES AT 35°C

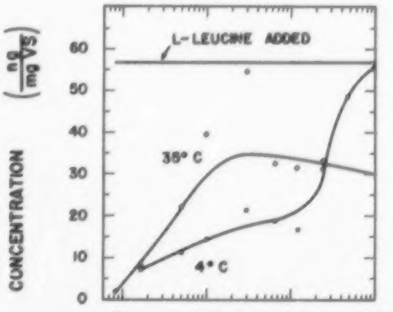


FIG. 6-15 UPTAKE AND UTILIZATION OF L-LEUCINE I-C-14 (4°C AND 35°C)

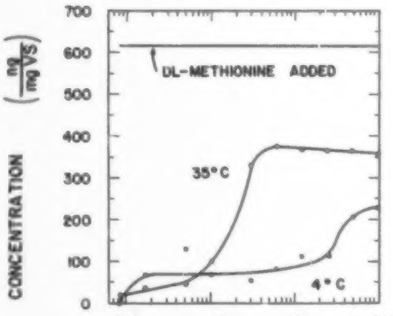


FIG. 6-18 UPTAKE AND UTILIZATION OF DL-METHIONINE I-C-14 (4°C AND 35°C)

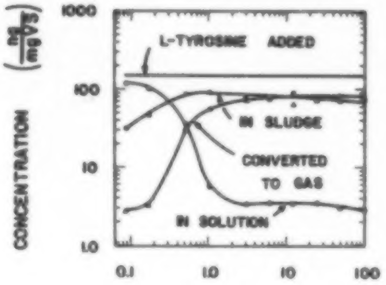


FIG. 6-19 DISTRIBUTION OF

L-TYROSINE 1-C-14 AT 35°C

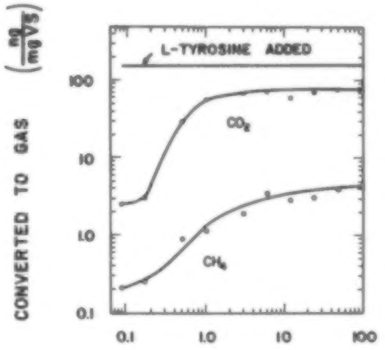


FIG. 6-20 CONVERSION OF L-TYROSINE

1-C-14 TO RADIOACTIVE GASES AT 35°C

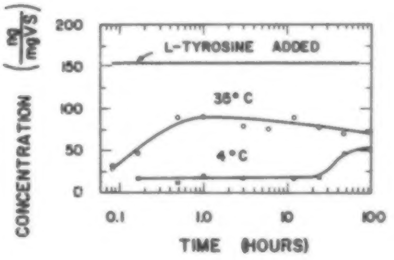


FIG. 6-21 UPTAKE AND UTILIZATION OF

L-TYROSINE 1-C-14 (4°C AND 35°C)

or 92.8 percent.

The effect of time on the production of carbon dioxide and methane gas is presented in Fig. 6-8. At five minutes, 0.607 ng of glycine per mg VS was converted to carbon dioxide and 0.098 ng of glycine per mg VS was converted to methane gas. After 96 hours these values increased to 14.07 and 0.700 ng, respectively.

The presence of any decomposition products of glycine containing the carbon-14 atom was established by means of paper chromatographic techniques. Samples of the solution were analyzed chromatographically at each of the time periods from time zero (standard) to 96 hours. The paper chromatograms were exposed to X-ray film. The radioautographs contained only glycine spots plus a trace at the origin. Subsequent measurement of the activity at the origin indicated that 2.50 cpm for the standard (time zero sample) was detectable while less than 1 cpm was detectable for the other samples. The data indicate that the small amount of activity remaining at the origin is glycine 1-C-14 hindered in its travel by the lead deposit left on the paper while drawing the circle of origin. It is possible that the lead itself contained enough natural carbon-14 to account for this activity. Therefore, a circle 3/8 inches in diameter was drawn on filter paper, completely blacked in with pencil lead. The quantity of pencil lead in this test was at least 100 times as great as the 1/8 inch diameter circle on the chromatogram. Measurement of the activity of this sample by the Sharp's Lowbeta did not yield any activity above background, thus indicating no measureable activity in the pencil lead. The data indicate that no decomposition product of glycine was present.

The data indicate that at the end of the 96 hour test period at 35°C, 1.52 ng of L-alanine 1-C-14 per mg VS, or 0.7 percent of the alanine added, remained in solution, 56.2 percent appeared as gas, and about 43.1 percent was associated with the sludge solids. After five minutes at 35°C, the total uptake (by the sludge mass) of L-alanine 1-C-14 was 41.1 ng per mg VS, or 18.1 percent of the total available L-alanine 1-C-14 of 226 ng per mg VS. The maximum uptake at 35°C was 196.6 ng per mg VS, or 87.0 percent at 1 hour. The maximum uptake rate at 35°C occurred between 0 and 5 minutes and was 493.2 ng per mg VS per hour. The maximum uptake at 4°C was 222.6 ng per mg VS, or 98.5 percent.

The effect of time on the production of carbon dioxide and methane gas is presented in Fig. 6-11. At five minutes, 2.77 ng of alanine per mg VS was converted to carbon dioxide and 0.43 ng of alanine per mg VS was converted to methane gas. At 96 hours, these values increased to 118.2 and 9.00, respectively, for carbon dioxide and methane.

The data indicate that no decomposition product of alanine was present.

The data indicate that at the end of the 96 hour test period at 35°C 0.105 ng of L-leucine 1-C-14 per mg VS, or 0.186 percent of the initial leucine concentration remained in solution, 46.9 percent appeared as gas, and about 52.9 percent was associated with the sludge solids. The total

uptake (by the sludge mass) of L-leucine 1-C-14 within five minutes at 35°C was 1.9 ng per mg VS or 3.36 percent of the total available L-leucine 1-C-14 of 56.6 ng per mg VS. The maximum uptake at 35°C was 54.5 ng per mg VS or 96.3 percent at three hours. This value and the 15.6 ng converted to gas per mg VS were greater than the total leucine 1-C-14 available of 56.6 ng per mg VS. Therefore, the curve was adjusted to yield a maximum uptake at 35°C of 34.0 ng per mg VS or 60.0 percent at three hours. The maximum uptake rate at 35°C occurred between 10 and 30 minutes and was 42.0 ng per mg VS per hour. The maximum uptake at 4°C was 55.6 ng per mg VS, or 98.2 percent. The production of carbon dioxide and methane gas with time of contact is shown in Fig. 6-14. At five minutes, 0.334 ng of leucine per mg VS was converted to carbon dioxide, and 0.069 ng of leucine per mg VS was converted to methane gas. At 12 hours, these values increased to 23.8 and 1.07, respectively, for carbon dioxide and methane.

The data indicate that no decomposition products of leucine were present.

The data indicate that at the end of the 96 hour test period at 35°C, 6.5 ng of DL-methionine 1-C-14 per mg VS, or 1.1 percent of the original methionine, remained in solution, 44.1 percent appeared as gas, and about 54.8 percent was associated with the sludge solids. Within five minutes at 35°C, the total uptake (by the sludge mass) of DL-methionine 1-C-14 was 19.4 ng per mg VS or 3.14 percent of the total available DL-methionine 1-C-14 of 617 ng per mg VS. The maximum uptake at

35°C was 372.8 ng per mg VS, or 60.5 percent, at six hours. The maximum uptake rate at 35°C occurred between 0 and 5 minutes and was 232.8 ng per mg VS per hour.

The production of carbon dioxide and methane gas with time of contact is shown in Fig. 6-17. At five minutes, 0.902 ng of methionine per mg VS was converted to carbon dioxide and 0.705 ng of methionine per mg VS was converted to methane gas. At 96 hours, this increased to 249 and 22.55, respectively, for carbon dioxide and methane.

The data indicate that no decomposition products of methionine were present.

The data indicate that at the end of the 96 hour test period at 35°C 2.88 ng of L-tyrosine 1-C-14 per mg VS, or 1.9 percent, remained in solution, 51.3 percent appeared as gas, and about 46.8 percent was associated with the sludge solids. After five minutes at 35°C, the total uptake (by the sludge mass) of L-tyrosine 1-C-14 was 32.0 ng per mg VS or 20.7 percent of the total available L-tyrosine 1-C-14 of 154.4 ng per mg VS. The maximum uptake at 35°C was 90.0 ng per mg VS, or 58.2 percent at one hour. The maximum uptake rate at 35°C occurred between 0 and 5 minutes and was 384.0 ng per mg VS per hour.

The effect of time on the production of carbon dioxide and methane gas is presented in Fig. 6-20. At five minutes, 2.605 ng of tyrosine per mg VS was converted to carbon dioxide and 0.213 ng of tyrosine per mg VS was converted to methane gas. At 96 hours, this increased to 75.0 and 4.21, respectively, for carbon dioxide and methane.

The data indicate that no decomposition products of tyrosine were present.

The gas production rates varied with the particular amino acid added as well as with the volatile solids concentration of the sludge substrate. Therefore, the rate of gas production is expressed in terms of nanoliters of gas resulting from the amino acid per milligram of volatile solids per hour (nl/mg VS/hr). These data are tabulated in Table A-15 in the Appendix.

The data indicate that L-alanine produced carbon dioxide gas at the highest rate and the rates of carbon dioxide gas production decreased respectively in the following order: DL-methionine, L-tyrosine, L-leucine, and glycine. The rate of conversion to methane was at a maximum with glycine and decreased in the following order: DL-methionine, L-alanine, L-tyrosine, and L-leucine. However, only a portion of the amino acid added is radioactive; therefore, the gas resulting from the breakdown of the added amino acid includes radioactive and non-radioactive components.

The data indicate that L-tyrosine produced labeled carbon dioxide gas at the highest rate: namely, 4.62 nl per mg VS per hour. The rates of labeled carbon dioxide gas production decreased respectively in the following order: L-alanine, L-leucine, glycine, and DL-methionine. However, the rate of conversion to labeled methane does not follow the same pattern. The maximum conversion to labeled methane was observed when L-alanine was added. L-tyrosine, DL-methionine, L-leucine, and glycine, respectively, caused lower rates of methane production.

The rate of uptake and utilization of L-alanine at 35°C was the highest of the amino acids added. The rates of L-tyrosine, DL-methionine, glycine, and L-leucine decreased respectively in this order.

The uptakes of glycine, L-alanine, and L-leucine at 4°C were greater than the uptake at 35°C. The uptake at 4°C is strictly a sorption process; therefore, an amino acid sorbed by the sludge mass can subsequently be released into solution and utilized by the microbial population.

CHAPTER 7

DISCUSSION

Anaerobic fermentation of organic material in wastewater sludges is a complex biological and biochemical process. Amino acids are common constituents of wastewater sludges and may be released during degradation of proteins. These compounds are necessary for the growth and metabolism of the microbial population responsible for the anaerobic stabilization of the organic materials. Hence, the uptake rate of amino acids should indicate the total metabolic activity in the anaerobic digestion unit. An understanding of the utilization, incorporation (uptake), and total turnover of amino acids during anaerobic digestion is necessary to improve the effectiveness of this process.

The effects of amino acids on the overall digestion process and the effects of the anaerobic environment in the digesters on amino acid utilization were the primary objectives of this study. The interaction and interrelationships of the amino acids and the environment are of prime concern.

7-1 EFFECTS OF AMINO ACIDS ON ANAEROBIC DIGESTION

Defined, dissolved media have been used in many of the recent studies in which anaerobic fermentation was evaluated by other investigators, but only a few studies employed primary sludge as a substrate. Primary sludge is not a well defined medium. Large variations in the concentration and composition of organic constituents and microbial flora of two laboratory units operating under the same conditions and to which the same primary sludge was added as feed are possible. Therefore, a discussion of the characteristics of the digesting sludge must be presented in conjunction with the amino acid uptake studies.

The data indicate that after the addition of an amino acid to the wastewater sludge in an anaerobic environment a slight increase in both alkalinity and volatile acid concentrations was observed but there was no significant change in pH; however, an increase in the free amino acid concentration was recorded. Localized conditions in an anaerobic digester are quite different from the average conditions; therefore, at a specific bacterial site the pH may be less than 7 thus allowing decarboxylation to occur, but the recorded pH for the overall system is near 8.

The data indicate that one of the reactions for these amino acids involved the release of ammonia. In the case of glycine, acetic acid would remain; therefore, an increase in volatile acid concentration was recorded. Amino acids at a concentration of less than 5 mM/l causes a small change in pH, alkalinity, and volatile acid concentration, but the gas production rate dropped to a much lower rate. However, gas production is only one measure of the degradation of organic compounds.

The gases formed during the anaerobic fermentation process under equilibrium conditions usually contain about 65 percent methane, 34 percent carbon dioxide, and about 1 percent nitrogen and other gaseous components. In the Warburg experiments in which non-radioactive glycine was added to the sludge inoculum, helium was used to flush the system. This atmosphere contained almost no carbon dioxide and methane gas. Therefore, a measure of the changes in the concentration of carbon dioxide and methane gas produced with time was possible. The data indicate that a ratio of 10 parts of carbon dioxide to 1 part of methane was maintained in all the anaerobic Warburg studies. This ratio was observed when wastewater sludge was the only material in the flask as well as when a labeled or non-radioactive amino acid was added to the sludge.

The low methane production compared to the carbon dioxide production in both the unlabeled and labeled amino acid studies of glycine may be explained by the fact that a reaction time of 96 hours was not sufficient for the methane gas concentration to increase to higher levels (131).

The effect of amino acid concentration on the gas production rates was evaluated after glycine was added to wastewater sludge. In one run 400 µl of gas were produced in excess of the gas produced by the sludge substrate alone after the addition of 1.08 mM/l of glycine. In the second run this increase was 950 µl (Table A-1).

The conditions of this experiment were similar to those used by Cardon (27) in which a 10-ml cell suspension and a 90-ml gas space were shaken continuously. Under these conditions the decomposition of glycine may be represented in the equation:

$$10\,CH_2(NH_2)COOH \;+\; 9.1\,H_2O \;\longrightarrow$$

$$10\,NH_3 \;+\; 5.3\,CH_3COOH \;+\; 9.3\,CO_2 \;+\; 8.6\,H_2$$

A glycine concentration of 1.08 mM/l in 15 ml of sludge gives a total of 16.2 μM of glycine available for reaction with the microorganisms. Applying the perfect gas law, the number of moles of carbon dioxide produced is:

$$PV = nRT$$

$$n = \frac{(1\ \text{atm})(400\ \mu l)}{(0.082\ l - \text{atm/mole} - {}^{\circ}C)(308^{\circ}C)}$$

$$n = 15.9\ \mu M \text{ of gas}$$

Theoretically 0.93 (16.2) = 15.1 μM of carbon dioxide can be produced (27). Since methane can arise from the reduction of carbon dioxide, the calculation for total carbon dioxide should represent the total gas shown on the curves (Fig. 6-1). On the basis of these calculations, the complete degradation of glycine added to the flask should yield about 380 μl of gas. This conversion of glycine to gaseous end products was accomplished in about 50 minutes. However, it must be borne in mind that there is no way in non-radioactive studies to establish whether the 400 μl additional gas produced was solely from the glycine.

The rate of gas production was measured with the Warburg respirometer. However, this technique does not distinguish between the gas resulting directly from the degradation of the amino acid and the gas resulting from other compounds in the sludge. Many organic compounds may be anaerobically converted to carbon dioxide and methane; therefore, radioactive tracer studies were necessary to evaluate the contribution to the gas production attributable directly to the degradation of the amino acids.

Glycine at a concentration of 1.08 mM/l had a stimulatory effect on gas production; however, at a concentration of 4 mM/l glycine was inhibitory to gas production. Therefore, a concentration less than 1 mM/l of a single labeled amino acid was added to the environment in the radioactive uptake studies.

The actual quantities of the 1-C-14 tagged amino acids added were 12.0 µM/l for glycine, 42.1 µM/l for L-alanine, 13.2 µM/l for L-leucine, 104.5 µM/l for DL-methionine, and 18.8 µM/l for L-tyrosine. These concentrations had essentially no effect on the environmental balance; however, an increase in the gas production rate resulted although the anaerobically digesting sludge contained a concentration of volatile solids of about 20,000 mg/l. A concentration of 12.0 µM/l of glycine 1-C-14 in 15 ml of sludge yields 0.180 µM of glycine available for degradation. Theoretically the complete breakdown of glycine to gaseous products would result in:

$$V = \frac{nRT}{P} = \frac{(0.180\ \mu M)(0.082\ l - atm/\mu M - {}^{\circ}C)(308^{\circ}C)}{1\ atm}$$

$$V = 4.53\ \mu l \text{ of gas}$$

The anaerobic degradation of glycine yields 0.93 moles of carbon dioxide per mole of glycine; hence 0.93 (4.53) = 4.22 µl of carbon dioxide gas should be produced (27). The experimental data indicate that for glycine gas was produced at a rate of 146 µl of gas per hour. These data indicate an increase of 67.7 µl per hour (Fig. 6-2). This increased rate of gas production continued for more than 10 minutes as shown by longer-term studies. A total of 70 µl of gas can be attributed directly to the breakdown of glycine. This amount is about 65 µl in excess of the theoretical maximum gas production from the available glycine 1-C-14. It is apparent from these data that glycine in micromole quantities has a stimulatory effect on gas production.

In the fermentation of alanine the overall reaction yields 1.5 moles of carbon dioxide per mole of alanine (27,61). But in the Strickland reaction the overall reaction of one mole of alanine with two moles of glycine yields four moles of carbon dioxide.

The addition of 0.63 µM of alanine to 15 ml of sludge results in a concentration of 42.1 µM/l and theoretically should yield about 15.9 µl of gas upon anaerobic degradation. However, each mole of alanine fermented results in 1.5 to 4.0 moles of carbon dioxide. Therefore, the total yield should be between 23.8 and 63.6 µl of carbon dioxide. The actual yield based on the data in Fig. 6-3 is about 220 µl. Similar calculations for leucine, methionine, and tyrosine also indicated that the actual amount produced in these studies was greater than the theoretical amount of carbon dioxide gas.

The specific amino acid may not be necessary in the metabolism of many bacteria; however, glycine, for example, may serve as a precursor in the production of needed compounds. Under these conditions it is apparent that the overall metabolism may increase by the presence of certain amino acids. It is apparent that the five amino acids had a stimulatory effect on gas production. Therefore, varying the amount of some amino acids in wastewater sludge affects the anaerobic microbial activity and hence the degree of conversion of the organic material to stable end products. Since amino acids are necessary in cellular metabolism, it is logical that increased microbial activity could result. If this is the case, it is possible that digestion efficiency could be predicted on the basis of the free amino acid concentrations.

7-2 EFFECTS OF ANAEROBIC DIGESTION ON THE AMINO ACID

Amino acids in an anaerobic environment are involved in a number of reactions. Amino acids may be decarboxylated or deaminated or both of these two degradation mechanisms may occur simultaneously. Tagging the number 1 carbon of an amino acid (i.e., the carboxyl carbon) yields radioactive carbon dioxide if the amino acid is decarboxylated. However, there seems to be no definite data for the enzymatic decarboxylation of glycine, alanine, and methionine to amines (104). If carbon dioxide is formed during the anaerobic decomposition of these amino acids, the products formed are not amines but are fatty acids, carbon dioxide, and ammonia. In the fermentation of glycine, for example, the carbon dioxide formed comes directly from the carboxyl group of glycine (15,27,41,124). Nitrogen tagging would yield information regarding the deamination of an amino acid. However, radioactive nitrogen labeled amino acids are not available since the half-life of the longest-lived nitrogen isotope is only 10 seconds. Amino acids with the stable nitrogen 15 are available, but the use of this isotope requires a mass spectrophotometer for analysis. Nitrogen 15 was not used at this time for this reason.

An alternate method for evaluating the effect of digestion on amino acids, although not as precise as radioactive techniques, involves a nitrogen balance based upon the Kjeldahl nitrogen test. This procedure involved estimating the concentration of ammonia nitrogen and organic nitrogen in both the total unaltered sludge sample and the supernatant obtained after separating the solids from the carrier liquid by centrifuging the samples at 21,000 x gravity.

If 1,000 mg/l of glycine is added to sludge, the Kjeldahl analysis of this mixture indicates an increase in organic nitrogen content of the total sample and of the supernatant of 186.5 mg/l of nitrogen. As this amino acid is utilized by the microbial population during the anaerobic digestion process, the concentration of organic nitrogen in the supernatant should decrease while the ammonia nitrogen in the supernatant should increase.

By using this Kjeldahl technique, it was possible to make a nitrogen balance and thus to establish that deamination does occur at a pH of approximately 8.0 in the anaerobic environment. If the pH is greater than 7, amino acids cannot be decarboxylated (31), hence deamination should be the only reaction. The data indicate that in a heterogeneous anaerobic wastewater environment tagged carbon dioxide is produced during the anaerobic degradation of an amino acid containing C^{14} in the carboxyl position.

It also should be pointed out that decarboxylation enzymes for glycine have not been isolated (47,52,53,150), but the data observed in this study indicate the production of carbon dioxide. The results of other investigations also demonstrate the production of carbon dioxide from glycine but acetic acid was an intermediate product (15,27,41,124,135, 136,137).

The data observed during the non-radioactive amino acid uptake studies indicate an increase in ammonia nitrogen concentration after the addition of glycine, alanine, methionine, and tyrosine in separate experiments (Table A-3). The data indicate an increase in organic nitrogen in the supernatant from the added amino acid but not equal to the amount added since there also is an increase in ammonia nitrogen in the supernatant (glycine only). High concentrations of glycine were used, thus making glycine a predominant organic nitrogen source and hence overruling other reactions.

Similar results were observed for alanine, methionine, and tyrosine; however, a lower concentration of each amino acid was used and hence the results are less predictable. It was noted that there was a slight decrease in the organic nitrogen content of the supernatant and a slight increase in ammonia nitrogen concentration in the supertatant. These data indicate that deamination is a major reaction with these amino acids. Inconclusive data was obtained with leucine.

The total quantity of low-weight molecular compounds that may be extracted from the bacterial cell under conditions that do not degrade macromolecules is defined as the "pool" (21).

Some bacteria, such as Escherichia coli, incorporate amino acids, but these amino acids are not free in the cell but are absorbed to larger molecules. Under this condition the amino acids can also diffuse out of the cell if they are not rapidly utilized or if they are not bound to cellular compounds.

The uptake and pooling of amino acids permits a more detailed discussion of the distribution of amino acid 1-C-14 in the gas, in solution, and associated with the sludge solids. These data observed for glycine 1-C-14 are presented in Figs. 6-7, 6-8, and 6-9. The free amino acid in solution approached zero and continued to decrease even though the value of the total uptake at 35°C also decreased at a more rapid rate.

This phenomenon can be explained as follows. A large portion of the amino acid was non-pooled and concentrated in the cell wall region with some of the amino acid adsorbed by the cell walls. Escherichia coli, for example, incorporate free amino acids, but these amino acids are not free in the cell but are absorbed to larger molecules; however, they retain the ability to diffuse out of the cell if they are not rapidly utilized or bound to cellular components. The non-pooled amino acid concentrated in the cell wall region and the absorption of amino acids to larger molecules can be classified as non-biological uptake. In this study, non-biological uptake is the uptake by sludge at 4°C. Hence, as the biological activity continued the free amino acid in solution decreased and some of the amino acid retained in a non-pooled state by the cells was released and made available to be biologically utilized by the cells. The net effect was a decrease in the measured total uptake and an increase in gas production.

The data presented in Fig. 6-9 indicate that the non-biological uptake (at 4°C) of glycine was greater than the uptake at 35°C. This information indicates that the capacity of the system, the sludge solids and bacteria, was capable of sorbing glycine at a rate which was in excess of the capacity of the system to degrade and utilize glycine.

The difference between the total uptake at 35°C and the non-biological uptake is not necessarily the biological uptake except possibly for the first few minutes (about the first 10 minutes). The conditions of the experiments at 35°C and at 4°C were entirely different; each attempted to show a different characteristic of the sludge mass. The non-biological uptake was performed with sludge acclimated at 4°C and was intended to show that amino acids can be bound to large molecules, bacterial cells, or to particulate material.

The total uptake was evaluated at 35°C under anaerobic conditions and included both the biological and non-biological uptake. Under these conditions, an amino acid that was originally bound to a molecule or particle may become available to the cell for utilization or degradation; therefore, the total uptake decreases. The total uptake at 35°C involves biological utilization or incorporation; therefore, the uptake may vary as the degradation of wastes and microbial metabolism progresses. The data indicate that some of the amino acid was converted to labeled carbon dioxide and labeled methane gas. Therefore, amino acids must be released from the sludge mass and converted to gas. These data explain why the non-biological uptake was greater than the total uptake for glycine (Fig. 6-9), alanine (Fig. 6-12), and leucine (Fig. 6-15).

The radioactive gas production rate varies considerably among the amino acids (Tables A-6, A-8, A-10, A-12, A-14, and A-15). The rate is represented in ng of amino acid converted to gas per mg VS per hour in order to be consistent with the other data involved in the amino acid materials balance. However, there is only one radioactive carbon atom per amino acid molecule which can result in radioactive carbon dioxide or methane molecules. The quantity of activity added to the sludge was 5 microcuries (μc) in all the experiments (except for glycine in which only 1 μc was added). However, the specific activity of each amino acid solution varied; therefore, the weight of each amino acid per unit of activity is as follows: (1) glycine, 9 nanograms/nanocurie (ng per nc), (2) L-alanine, 11.24 ng per nc, (3) L-leucine, 5.19 ng per nc, (4) DL-methionine, 46.7 ng per nc, and (5) L-tyrosine, 6.82 ng per nc.

One μc of activity originates from 0.218 μg of C^{14}. Therefore, the activity of pure C^{14} is 64 μc per μM. The specific activity of the alanine solution used in this experiment was 8.00 μc per μM; that is, 8.00 out of every 64 alanine molecules was radioactive. Therefore, 1 nc of activity in the gas results from the degradation of 1.4 ng of labeled alanine.

Five μc of alanine were added to 15 ml sludge which contained a volatile solids concentration of 16.575 μg per μl. Therefore, the total available alanine supplied to the sludge was 226 ng/mg VS. This value includes alanine 1-C-14 and non-radioactive alanine. Detailed calculations are included in the Appendix in connection with Table A-16. The variables which must be remembered in evaluating the degradation of 1-C-14 amino acids are:

1) Only a portion of the amino acid molecules are radioactive;
2) The molecular weights of each amino acid; and
3) The volatile solids concentration of the sludge.

Because of these variables the gas production data are presented in ng of amino acid converted to gas per mg VS and are useful in the materials balance of an individual amino acid. The gas production rate in ng of amino acid converted to gas per mg VS per hour cannot be used to compare the quantity of the individual constituents of the gas resulting from different amino acids. Therefore, the maximum rates of gas production have been converted to µl of gas per hour (Table A-15). Sample calculations are presented in Table A-16.

The data indicate that on a molar basis there was a negligible difference in the maximum rate of gas production among the monoamino-monocarboxylic acids glycine, L-alanine, and L-leucine, namely 9.34, 7.64, and 9.04 µl/µM/hr, respectively. The sulfur amino acid, DL-methionine, produced gas at a rate of 1.88 µl/µM/hr, but only the L form of methionine is utilized microbially. L-tyrosine produced gas at a rate of 13.33 µl/µM/hr. However, the effect of each of these amino acids on the overall environment was considerable (Table 6-2, Fig. 6-2 through 6-6). The data indicate that there was no direct relationship between amino acid gas production rate and the stimulatory rate of gas production.

Amino acids in micromole quantities have a stimulatory effect on the anaerobic digestion process; therefore, the control of this parameter may be a means of increasing the effectiveness of the digestion process.

The biological reduction of tagged carbon dioxide to methane was observed, but the rate of methane production was low in comparison with the rate of carbon dioxide production. An explanation of these results follows.

The bacterial degradation of one mole of acetate yields one mole of carbon dioxide and one mole of methane; however, the carbon dioxide results from the carboxyl carbon (117,118,131) and only a very small portion of this carbon dioxide is further reduced to methane.

In an anaerobic digester carbon dioxide and methane are regularly produced. However, with glycine 1-C-14, tagged methane can only come from the carboxyl carbon of glycine. The results of many experiments have shown that the reduction of carbon dioxide to methane is a minor reaction.

Tagged methane can also be formed through other reactions.

The fermentation of unlabeled valerate in the presence of labeled carbon dioxide by Methanobacterium suboxydans is as follows (132,133):

$$2\,CH_3(CH_2)_3COOH + 2\,H_2O + C^{14}O_2 \longrightarrow$$

$$2\,CH_3CH_2COOH + 2\,CH_3COOH + C^{14}H_4$$

The fermentation of unlabeled butyrate in the presence of labeled carbon dioxide by Methanobacterium suboxydans yields acetic acid and labeled methane (132,133):

$$2\,CH_3CH_2CH_2COOH + 2\,H_2O + C^{14}O_2 \rightarrow$$

$$4\,CH_3COOH + C^{14}H_4$$

The fermentation of propionate by Methanobacterium propionicum yields the following reaction (132,133):

$$4\,CH_3CH_2COOH + 2\,H_2O + 4\,C^{14}O_2 + 24\,H \rightarrow$$

$$4\,CH_3COOH + CO_2 + C^{14}O_2 + 3\,CH_4 + 3\,C^{14}H_4 + 6\,H_2O$$

The reactions offer an explanation of the mechanism of producing tagged methane gas. A plentiful supply of these particular intermediate fatty acids is usually available during the anaerobic digestion of wastewater sludge; therefore, more methane than had been observed after a 96-hour test period would have been expected.

The anaerobic degradation of amino acids has been reported by other investigators and some of the data recorded in the studies reported herein may be explained by various reactions and degradation schemes.

A minor fermentation reaction of glycine results in the formation of acetate, carbon dioxide, and ammonia; however, the acetate may have been immediately converted to carbon dioxide (24,27,41,117,118,124,131).

Free amino acids were detected in the digesting sludge. Therefore, it is possible that glycine could participate with alanine or another hydrogen donor in a Strickland reaction (oxidative deamination)(135,136,137,142, 143,144,145) and only acetate and ammonia will result from glycine. The carboxyl carbon of acetate is tagged if glycine 1-C-14 is added and upon degradation of the acetate labeled carbon dioxide is released. Glycine may also be involved in a reductive deamination reaction which leads to the formation of saturated fatty acids (150).

The data from the radioautographs indicate that labeled intermediate products were not detectable in samples withdrawn from the anaerobic environment containing wastewater sludge. The results of other radioactive studies have shown that glycine degraded anaerobically is deaminated (27, 41,124).

Two basic anaerobic degradation reactions have been reported for alanine. The first reaction is the conversion of alanine 1-C-14 to propionate 1-C-14 by the organism *Clostridium propionicum* (26,27,61). In this degradation scheme three moles of alanine resulted in two moles of propionic acid, one mole of acetic acid, one mole of carbon dioxide, and three moles of ammonia. The further degradation of propionate 1-C-14 by *Methanobacterium propionicum* yields unlabeled acetic acid and labeled carbon dioxide. This labeled carbon dioxide can react with additional propionate to yield labeled methane (132,133,134). This series of reactions would result in labeled carbon dioxide and methane.

The second possible degradation scheme is a mutual oxidation-reduction between two amino acids (a Strickland reaction) resulting in deamination of both amino acids. The hydrogen donor in this reaction is alanine; glycine, for example, may serve as the hydrogen acceptor. It is postulated that the reaction of alanine 1-C-14 with glycine would proceed as follows:

$$CH_3CH(NH_2)C^{14}OOH \quad + \quad 2\ CH_2(NH_2)COOH \quad + \quad 2\ H_2O \quad \longrightarrow$$

$$2\ CH_3COOH \quad + \quad CH_3C^{14}OOH \quad + \quad 3\ NH_3 \quad + \quad CO_2$$

Clostridium sporogenes and several other species of the genus *Clostridium* participate in this reaction (27,142,143,144,145). The acetate 1-C-14 is degraded by methane-producing organisms such as *Methanococcus* to labeled carbon dioxide (Chapter 2) (24,117,118,131).

The only evidence of anaerobic microbial degradation of leucine is by means of a mutual oxidation-reduction reaction between two amino acids (the Strickland reaction) (oxidative deamination) (104). L-leucine participates as a hydrogen donor in the Strickland reaction which is catalyzed by the organism *Clostridium sporogenes* and some other species of this genus (142,143,144,145,163). In the anaerobic reaction between L-leucine and

L-proline (a hydrogen acceptor), isovaleric acid and carbon dioxide are formed. The carbon dioxide originates from the number 2 carbon. Tagging the number 1 carbon of leucine results in the formation of isovaleric acid 1-C-14. Assuming that beta-oxidation (Chapter 2) occurs during the degradation of isovaleric acid, acetate 1-C-14 is released. Further degradation of acetate 1-C-14 yields labeled carbon dioxide. Isovaleric acid is utilized as rapidly as it is formed with the release of tagged carbon dioxide since no product other than leucine was positively identified by radioautography. Leucine is not decarboxylated during the Strickland reaction; however, ammonia is released.

Methionine is fermented by certain strains of Pseudomonas to yield ammonia, α-keto butyric acid, and methyl mercaptan (77). The carboxyl group of α-keto butyric acid would contain C^{14} from the tagged carboxyl group of methionine. The α-keto butyric acid therefore would yield carbon dioxide. It is postulated that if methionine 1-C-14 is degraded anaerobically the reaction would proceed as follows:

$$CH_3SCH_2CH_2CH(NH_2)C^{14}OOH \; + \; H_2O \; \longrightarrow$$

$$CH_3CH_2COC^{14}OOH \; + \; H_3CSH \; + \; NH_3$$

$$\Big\downarrow \swarrow H_2O$$

$$CH_3C^{14}OOH \; + \; CH_3COOH$$

The acetate 1-C-14 is then degraded by a methane organism such as Methanococcus to yield labeled carbon dioxide (24,117,118,131).

There is no evidence which indicates that tyrosine undergoes anaerobic fermentation. However, tyrosine can be deaminated, decarboxylated, and participate in some microbial transamination reactions. The results of the investigation presented herein indicate that carbon dioxide is formed during tyrosine degradation, but a breakdown scheme cannot be established.

CHAPTER 8

CONCLUSIONS

1) The micromole quantities of glycine, L-alanine, L-leucine, DL-methionine, and L-tyrosine added to digesting sludge have a stimulatory effect on the total gas production rate of the anaerobic system. Gas production is stimulated to the greatest extent by tyrosine, followed in order by leucine, methionine, alanine, and glycine, respectively. One millimole per liter of unlabeled glycine increased the rate of gas production, but at a concentration of 4 millimoles per liter glycine exhibited an inhibitory effect on the gas production during anaerobic digestion.

2) Control of the amino acid concentration in a digester may be a means of increasing the effectiveness of the anaerobic digestion process. The digester efficiency may be predicted on the basis of amino acid concentration since amino acids can stimulate or inhibit gas production.

3) Glycine, alanine, and methionine were not decarboxylated enzymatically to amines. However, intermediate degradation products of these amino acids are decarboxylated to yield labeled carbon dioxide and subsequently labeled methane. Leucine and tyrosine also yield labeled carbon dioxide and labeled methane.

4) Deamination of the amino acids was one of the principle degradation mechanisms. Glycine, alanine, leucine, methionine, and tyrosine resulted in an increase in concentration of ammonia nitrogen during anaerobic digestion of wastewater sludge.

5) The monoamino-monocarboxylic amino acids glycine, leucine, and alanine are sorbed and pooled by the sludge mass to a greater extent than methionine and tyrosine. Sorption at 4°C by the anaerobic wastewater sludge mass was greater than the sorption at 35°C for glycine, alanine, and leucine. Micromole quantities of amino acids can be readily utilized by the anaerobic microbial population responsible for digestion of wastewater sludge.

6) The uptake of amino acids is a function of the biological and chemical characteristics of the sludge, the particular amino acid, the amino acid concentration, and the temperature of the environment.

APPENDIX A

Table A-1

Gas Production after Addition of Non-Radioactive Glycine (Warburg Respirometer, Temperature 35°C)

Concentration of glycine added mM/l	Run 1			Run 2		
	0	1.08	4.0	0	1.08	4.0
Time Minutes	**Gas Produced microliters**			**Gas Produced microliters**		
0	0	0	0	0	0	0
10	2300	3000	2400	2450	2550	2400
20	3200	3950	3150	3200	3500	3150
30	3800	4550	3650	3750	4100	3650
40	4300	5060	4000	4200	4560	4000
50	4650	5450	4340	4550	4950	4340
60	5000	5780	4600	4900	5300	4600
70	5250	6100	4890	5150	5550	4850
80	5550	6350	5120	5400	5850	5060
90	5750	6600	5350	5625	6060	5220
100	5975	6840	5550	5850	6270	5380
110	6150	7050	5740	6050	6460	5520
120	6350	7250	5900	6225	6650	5620
130	6540	7450	6100	6400	6800	5750
140	6700	7650	6250	6550	6950	5850
150	6850	(a)	6400	6725	7100	5940
160	7000	(a)	6550	6870	7260	6050
170	7150	(a)	6700	7000	7400	6150
180	7300	(a)	6820	7140	7550	6240

(a) Data not recorded.

Table A-2

Physical and Chemical Characteristics of Sludge Samples Used in the Evaluation of Amino Acid Uptake and Utilization

Description of Sample	Sample Number 1	 2 Glycine added	 3	 4 Glycine added	 5	 6 Glycine added	 7 Feed for Samples 3 - 6
pH	7.8	8.0	8.0	8.1	8.2	8.0	6.5
Total Solids, mg/l	34,100	(a)	58,900	53,400	52,000	51,500	62,500
Volatile Solids, mg/l	22,400	(a)	33,950	31,900	30,475	31,100	47,650
Alkalinity, mg/l as Calcium Carbonate	3,230	3,180	2,750	2,700	3,000	3,580	1,470
Volatile Acids, mg/l as Acetic Acid	433	696	912	744	300	420	1,478
Ammonia Nitrogen, mg/l as Nitrogen	783	875	552	865	985	1,120	395
Organic Nitrogen, mg/l as Nitrogen	952	1,435	1,650	1,920	1,460	1,901	1,928
Total Nitrogen, mg/l as Nitrogen	1,735	2,310	2,202	2,785	2,446	3,021	2,323
Proteins, mg/l as Bovine Serum Albumin	31,900	30,570	14,670	12,500	13,200	13,240	14,840
Amino Acids, mg/l as Glycine	(a)	6,750	269	3,070	490	2,725	530
Lipids, mg/l	3,690	4,120	6,250	5,600	9,590	5,920	(a)
Carbohydrates, mg/l as Glucose	2,286	2,865	11,800	11,725	4,078	4,194	14,970
Total Carbon, mg/l as Carbon	10,240	12,800	25,600	22,100	18,450	19,160	28,350

Table A-2 (Continued)

Description of Sample	Sample Number						
	8	9 Glycine added	10 Feed for Samples 8 and 9	11	12 Glycine added	13 Feed for Samples 11 and 12	14
pH	8.0	8.4	6.3	8.1	8.1	6.2	7.8
Total Solids, mg/l	49,000	45,950	50,450	40,900	40,600	55,000	35,050
Volatile Solids, mg/l	30,650	29,950	33,100	26,620	26,290	42,750	21,650
Alkalinity, mg/l as Calcium Carbonate	3,050	3,560	1,620	3,340	3,780	1,200	3,710
Volatile Acids, mg/l as Acetic Acid	216	312	2,360	144	216	1,800	705
Ammonia Nitrogen, mg/l as Nitrogen	811	903	224	761	845	455	784
Organic Nitrogen, mg/l as Nitrogen	1,327	1,763	2,840	1,245	1,510	1,441	1,325
Total Nitrogen, mg/l as Nitrogen	2,138	2,666	3,064	2,006	2,355	1,896	2,109
Proteins, mg/l as Bovine Serum Albumin	9,500	9,000	12,950	12,675	13,120	19,000	9,280
Amino Acids, mg/l as Glycine	827	4,423	837	916	1,348	637	1,160
Lipids, mg/l	6,520	5,200	14,920	3,980	4,040	11,000	4,350
Carbohydrates, mg/l as Glucose	5,285	4,160	18,700	4,480	2,820	18,700	4,500
Total Carbon, mg/l as Carbon	14,950	16,650	23,200	15,000	15,500	25,850	14,225

Table A-2 (Continued)

Description of Sample	Sample Number						
	15 Glycine added	16 Feed for Samples 14 and 15	17	18 Alanine added	19 Glycine 1-C-14	20 Feed for Samples 17 - 28	21 Alanine 1-C-14
pH	8.1	6.8	8.0	8.0	8.1	6.9	7.9
Total Solids, mg/l	32,125	47,400	31,150	43,300	46,350	44,600	41,275
Volatile Solids, mg/l	20,050	33,500	16,825	22,500	24,950	32,520	16,575
Alkalinity, mg/l as Calcium Carbonate	4,060	1,240	3,410	4,310	2,870	970	3,050
Volatile Acids, mg/l as Acetic Acid	865	1,465	120	228	780	960	228
Ammonia Nitrogen, mg/l as Nitrogen	889	141	1,009	1,321	678	267	583
Organic Nitrogen, mg/l as Nitrogen	1,438	1,331	1,458	1,140	1,260	1,266	1,020
Total Nitrogen, mg/l as Nitrogen	2,327	1,472	2,467	2,461	1,938	1,533	1,603
Proteins, mg/l as Bovine Serum Albumin	(a)	12,835	9,200	9,700	11,100	11,350	8,325
Amino Acids, mg/l as Glycine	(a)	900	978	713	311	200	447
Lipids, mg/l	(a)	9,150	2,270	2,720	5,720	7,135	1,960
Carbohydrates, mg/l as Glucose	(a)	34,400	2,040	2,560	3,500	10,000	3,700
Total Carbon, mg/l as Carbon	(a)	23,800	11,075	11,580	14,480	19,125	10,590

Table A-2 (Continued)

Description of Sample	Sample Number						
	22	23 Leucine added	24 Leucine 1-C-14	25 Methionine 1-C-14	26 Methionine added	27 Tyrosine 1-C-14	28 Tyrosine added
pH	8.1	8.1	8.0	8.0	8.1	8.0	8.0
Total Solids, mg/l	46,350	41,900	56,620	44,200	39,500	36,620	32,030
Volatile Solids, mg/l	24,950	23,450	30,560	25,275	21,650	22,080	20,360
Alkalinity, mg/l as Calcium Carbonate	2,870	3,040	3,290	3,130	3,340	1,250	1,330
Volatile Acids, mg/l as Acetic Acid	780	684	324	120	180	48	48
Ammonia Nitrogen, mg/l as Nitrogen	678	553	860	716	843	687	665
Organic Nitrogen, mg/l as Nitrogen	1,260	1,210	1,517	1,432	1,029	1,048	1,075
Total Nitrogen, mg/l as Nitrogen	1,938	1,763	2,377	2,148	1,872	1,735	1,740
Proteins, mg/l as Bovine Serum Albumin	11,100	12,000	13,750	13,775	11,400	10,000	12,750
Amino Acids, mg/l as Glycine	311	332	612	1,007	1,320	217	860
Lipids, mg/l	5,720	6,420	4,230	2,485	4,000	2,700	1,990
Carbohydrates, mg/l as Glucose	3,500	3,140	5,800	4,160	3,520	4,935	6,375
Total Carbon, mg/l as Carbon	14,480	14,500	17,700	12,100	12,540	12,660	12,210

(a) Data not recorded.

Table A-3

Ammonia Production after the Addition of Non-Radioactive Amino Acids

Sample Number	Nitrogen Added mg/l as N	Sludge			Supernatant		
		Ammonia Nitrogen mg/l as N	Organic Nitrogen mg/l as N	Total Nitrogen mg/l as N	Ammonia Nitrogen mg/l as N	Organic Nitrogen mg/l as N	Total Nitrogen mg/l as N
1	560	783	952	1735	640	67	707
2 (Glycine)(a)		875	1435	2310	627	571	1198
Difference		92	483	575	-13	504	491
3	560	552	1650	2202	677	61	738
4 (Glycine)(b)		865	1920	2785	809	553	1362
Difference		313	270	583	132	492	624
5	560	986	1460	2446	711	53	764
6 (Glycine)(b)		1120	1901	3021	873	558	1431
Difference		134	441	575	162	505	667
8	560	811	1327	2138	775	48	823
9 (Glycine)(d)		903	1763	2666	805	576	1381
Difference		92	436	528	30	528	558
11	374	761	1245	2006	732	64	796
12 (Glycine)(b)		845	1510	2355	813	33	846
Difference		84	265	349	81	-31	50

Table A-3 (Continued)

Sample Number	Nitrogen Added mg/l as N	Sludge			Supernatant		
		Ammonia Nitrogen mg/l as N	Organic Nitrogen mg/l as N	Total Nitrogen mg/l as N	Ammonia Nitrogen mg/l as N	Organic Nitrogen mg/l as N	Total Nitrogen mg/l as N
14	187	784	1325	2109	752	39	791
15 (Glycine)[b]		889	1438	2327	888	336	1224
Difference		105	113	218	136	297	433
17	70	1009	1458	2467	880	77	957
18 (Glycine)[e]		1321	1140	2461	940	49	989
Difference		312	-318	-6	60	-28	32
25	70	716	1432	2148	754	46	800
26 (Methionine)[c]		843	1029	1872	760	70	830
Difference		127	-303	-176	6	24	30
27	68	687	1048	1735	616	35	651
28 (Tyrosine)[c]		665	1075	1740	658	61	719
Difference		-22	27	5	42	26	68

(a) Sample 2 was withdrawn 2.5 hours after the amino acid was added to the digesting sludge of Sample 1.

(b) Samples 4, 6, 12, and 15 were withdrawn 24 hours after the amino acid was added to the digesting sludge of Samples 3, 5, 11, and 14, respectively.

(c) Samples 26 and 28 were withdrawn 48 hours after the amino acid was added to the digesting sludge of Samples 25 and 27, respectively.

(d) Sample 9 was withdrawn 72 hours after the amino acid was added to the digesting sludge of Sample 8.

(e) Sample 18 was withdrawn 120 hours after the amino acid was added to the digesting sludge of Sample 17.

(f) Sample numbers apply to Tables A-2, A-3, and A-4.

Table A-4

Carbon Balance

Sample Number (a) (1)	Feed Rate ml/day (2)	Actual Digester Volume liters (3)	Total Carbon in Dissolved Organic Compounds (b) mg Carbon (4)	Total Carbon in Gas Produced mg Carbon (5)	Total Carbon in Digester Sludge (c) mg Carbon (6)	Inorganic Carbon (Col. 6 - Col. 4) mg Carbon (7)	Unaccountable Carbon, Feed-(Col. 6 + Col. 5) mg Carbon (8)
11	500	6	5,441	4,870	7,500	2,059	555
12	500	6	5,349	6,170	7,750	2,401	-995
13 (feed)	-	-	12,412	-	12,925	513	-
14	400	6	3,980	5,820	5,680	1,700	-1,980
15	400	6	4,070	3,930	5,390	1,320	200
16 (feed)	-	-	10,800	-	9,520	-1,280	-
20 (feed)	-	-	5,860	-	7,650	1,790	-
19	400	6	4,400	2,320	5,780	1,380	-450
17	400	5.5	2,850	209	4,430	1,580	3,011
18	400	5.5	3,130	0	4,630	1,500	3,020
21	400	5	2,810	3,970	4,230	1,420	-550
20 (feed)	-	-	5,860	-	7,650	1,790	-
22	400	6	4,400	2,320	5,780	1,380	-450
23	400	6	4,650	1,870	5,850	1,170	-70
24	400	6	4,800	4,180	7,080	2,280	3,610
25	400	5.5	4,100	3,750	4,840	740	-940
26	400	5.5	4,460	2,040	5,020	560	590
27	400	5.5	3,450	3,810	5,060	1,610	-1,220
28	400	5.5	4,090	1,920	4,890	800	840

(a) See Table A-2.
(b) Based on theoretical carbon content of lipids 70%, carbohydrates 40%, proteins 45%, amino acids 45%, volatile acids 42%, cells 52%.
(c) Measured by the Van Slyke-Folch method (155).

Table A-5

Glycine 1-C-14 Uptake and Utilization

Contact Time Hours	Free Amino Acid in the Supernatant (35°C) $\frac{ng}{mg\ VS}$ (1)	Amino Acid Converted to Gas (35°C) $\frac{ng}{mg\ VS}$ (2)	Amino Acid Taken up by the Sludge (35°C) $\frac{ng}{mg\ VS}$ (3)	Non-biological Uptake (4°C) $\frac{ng}{mg\ VS}$ (4)
0	36.1	0	0	0
0.0833	19.1	0.705	16.3	2.4
0.1667	12.4	1.83	21.9	5.8
0.50	2.69	3.02	30.4	5.1
1	2.31	2.93	30.9	7.0
3	2.15	3.70	30.3	11.2
6	2.28	2.03	31.8	22.7
12	1.14	4.04	30.9	32.7
24	1.33	4.55	30.2	33.1
48	1.04	9.23	25.8	33.5
96	0.86	14.76	20.5	33.5

(a) Total amount of glycine 1-C-14 added = 36.1 ng/mg VS.
(b) 1.0 μc of glycine 1-C-14 added to 10 ml of sludge.
(c) Activity of glycine 1-C-14 added = 9.00 ng/nc.
(d) 1.0 μl of sludge = 24.950 μg VS.
(e) ng = nanograms = 10^{-9} grams.
(f) μl = microliter = 10^{-6} liters.
(g) μc = microcurie = 10^{-6} curies.
(h) ml = milliliter = 10^{-3} liters.
(i) VS = volatile solids.

Table A-6

Gas Production and Composition, Glycine 1-C-14 Added

Contact Time	Radioactive Gas Production			Percent of Total Applied Activity Appearing as Gas	Amino Acid Converted to Gas $\frac{ng}{mg\ VS}$			Rate of Amino Acid Converted to Gas During Increment $\frac{ng}{mg\ VS\ hr}$		
Hours (1)	CH_4 nc (2)	CO_2 nc (3)	Total nc (4)	(5)	CH_4 (6)	CO_2 (7)	Total (8)	CH_4 (9)	CO_2 (10)	Total (11)
0	0	0	0	0	0	0	0	0	0	0
0.0833	2.72	16.82	19.54	1.954	0.098	0.607	0.705	1.176	7.284	8.460
0.1667	3.30	47.25	50.55	5.055	0.120	1.70	1.82	0.264	13.116	13.380
0.50	2.68	81.0	83.68	8.37	0.096	2.92	3.02	-0.06	3.66	3.60
1	4.22	77.0	81.22	8.12	0.153	2.78	2.93	0.114	-0.28	-0.16
3	5.87	96.6	102.47	10.25	0.215	3.48	3.70	0.032	0.35	0.385
6	4.30	51.7	56.00	5.60	0.155	1.86	2.02	-0.020	-0.54	-0.56
12	≈4	108.2	≈112	11.20	≈0.13	3.91	4.04	-0.003	0.34	0.337
24	3.72	122.3	126.0	12.60	0.134	4.42	4.55	0.0	0.043	0.043
48	9.27	247	256.3	25.63	0.334	8.91	9.23	0.008	0.187	0.195
96	19.39	390	409.4	40.94	0.700	14.07	14.76	0.008	0.107	0.115

(a) Sample calculation for conversion of Column 2 to Column 6 at 0.0833 hours

$$\text{Col. } 6 = \frac{2.72\ \text{nc}}{10\ \text{ml}} \times \frac{\text{ml}}{24.950\ \text{mg}} \times \frac{9.00\ \text{ng}}{\text{nc}} = \frac{0.098\ \text{ng}}{\text{mg VS}}$$

(b) Sample calculation for conversion of Column 6 to Column 9 at 0.0833 hours

$$\text{Col. } 9 = \frac{(0.098 - 0)\ \text{ng}}{\text{mg VS}} \times \frac{1}{(0.0833 - 0)\ \text{hr}} = \frac{1.176\ \text{ng}}{\text{mg VS hr}}$$

Table A-7

L-Alanine 1-C-14 Uptake and Utilization

Contact Time Hours	Free Amino Acid in the Supernatant (35°C) ng/mg VS (1)	Amino Acid Converted to Gas (35°C) ng/mg VS (2)	Amino Acid Taken up by the Sludge (35°C) ng/mg VS (3)	Non-biological Uptake (4°C) ng/mg VS (4)
0	226	0	0	0
0.0833	181.7	3.2	41.1	32.3
0.1667	160.0	2.0	64.0	23.7
0.50	117.6	24.8	83.6	27.0
1	9.25	20.1	196.6	30.5
3	4.3	29.3	192.4	61.7
6	2.97	110.7	112.3	88.8
10	2.51	140.2	83.3	96.7
24	2.02	123.7	100.3	143.8
48	1.98	126.8	97.2	211.7
96	1.52	127.2	97.3	222.6

(a) Total amount of L-alanine 1-C-14 added = 226 ng/mg VS.
(b) 5.0 μc of L-alanine 1-C-14 added to 15 ml of sludge.
(c) Activity of L-alanine 1-C-14 added = 11.237 ng/nc.
(d) 1.0 μl of sludge = 16.575 μg VS.

Table A-8

Gas Production and Composition, L-Alanine 1-C-14 Added

Contact Time	Radioactive Gas Production			Percent of Total Applied Activity Appearing as Gas	Amino Acid Converted to Gas $\frac{ng}{mg\ VS}$			Rate of Amino Acid Converted to Gas During Increment $\frac{ng}{mg\ VS\ hr}$		
Hours (1)	CH_4 nc (2)	CO_2 nc (3)	Total nc (4)	(5)	CH_4 (6)	CO_2 (7)	Total (8)	CH_4 (9)	CO_2 (10)	Total (11)
0	0	0	0	0	0	0	0	0	0	0
0.0833	9.45	61.2	70.65	1.41	0.43	2.77	3.21	5.16	33.24	38.40
0.1667	13.95	30.4	44.35	0.89	0.63	1.37	2.00	2.40	-16.80	-14.40
0.50	30.6	517	547.6	10.95	1.38	23.4	24.8	2.25	66.09	68.40
1	42.6	402	444.6	8.90	1.92	18.22	20.14	1.08	-10.36	-9.32
3	52.9	594	646.9	12.95	2.40	26.85	29.25	0.24	4.31	4.55
6	80.0	2365	2445	48.9	3.62	107.0	110.7	0.41	26.72	27.15
10	106.8	2990	3097	61.9	4.83	135.3	140.2	0.30	7.07	7.37
24	99.9	2635	2735	54.7	4.52	119.2	123.7	-0.022	-1.15	-1.17
48	170.8	2635	2806	56.1	7.72	119.2	126.8	0.133	0.0	0.133
96	199.0	2615	2814	56.3	9.00	118.2	127.2	0.026	-0.020	0.006

Table A-9

L-Leucine 1-C-14 Uptake and Utilization

Contact Time Hours	Free Amino Acid in the Supernatant (35°C) ng/mg VS (1)	Amino Acid Converted to Gas (35°C) ng/mg VS (2)	Amino Acid Taken up by the Sludge (35°C) ng/mg VS (3)	Non-biological Uptake (4°C) ng/mg VS (4)
0	56.6	0	0	0
0.0833	54.3	0.402	1.9	-
0.1667	47.3	0.971	8.33	7.3
0.50	29.9	4.42	22.3	11.3
1	2.31	14.8	39.7	14.3
3	0.572	1.56	54.5	21.1
6.5	0.254	24.0	32.4	18.8
12	0.286	24.9	31.4	16.7
24	0.131	23.1	33.4	31.1

(a) Total amount of L-leucine 1-C-14 added = 56.6 ng/mg VS.
(b) 5.0 μc of L-leucine 1-C-14 added to 15 ml of sludge.
(c) Activity of L-leucine 1-C-14 added = 5.19 ng/nc.
(d) 1.0 μl of sludge = 30.560 μg VS.

Table A-10

Gas Production and Composition, L-Leucine 1-C-14 Added

Contact Time	Radioactive Gas Production			Percent of Total Applied Activity Appearing as Gas	Amino Acid Converted to Gas $\frac{\text{ng}}{\text{mg VS}}$			Rate of Amino Acid Converted to Gas During Increment $\frac{\text{ng}}{\text{mg VS hr}}$		
Hours (1)	CH_4 nc (2)	CO_2 nc (3)	Total nc (4)	(5)	CH_4 (6)	CO_2 (7)	Total (8)	CH_4 (9)	CO_2 (10)	Total (11)
0	0	0	0	0	0	0	0	0	0	0
0.0833	6.10	29.6	35.7	0.71	0.069	0.334	0.402	0.828	4.008	4.830
0.1667	5.54	80.6	86.14	1.72	0.0628	0.908	0.971	-0.074	6.888	6.828
0.50	15.1	377	392	7.85	0.1708	4.24	4.42	0.324	9.996	10.347
1	47.4	1243	1290	25.8	0.536	14.06	14.58	0.730	19.64	20.32
3	76.7	-	-	-	0.867	-	-	0.165	-	-
6.5	88.2	2035	2123	42.5	0.997	23.0	24.0	0.092	1.788	1.884
12	94.2	2105	2199	44.0	1.07	23.8	24.9	0.012	0.133	0.150
24	139.6	1900	2040	40.7	1.585	21.5	23.1	0.043	-0.096	-0.075

Table A-11

DL-Methionine 1-C-14 Uptake and Utilization

Contact Time Hours	Free Amino Acid in the Supernatant (35°C) $\frac{ng}{mg\ VS}$ (1)	Amino Acid Converted to Gas (35°C) $\frac{ng}{mg\ VS}$ (2)	Amino Acid Taken up by the Sludge (35°C) $\frac{ng}{mg\ VS}$ (3)	Non-biological Uptake (4°C) $\frac{ng}{mg\ VS}$ (4)
0	617	0	0	0
0.0833	596	1.6	19.4	0
0.1667	577	3.6	36.4	69
0.50	562	9.2	45.8	130
1	493	24.6	99.4	69
3	176	112.5	328.5	55
6	7.2	237	372.8	82
12	5.8	243	368.2	114
25.5	6.35	245	365.6	115
48.5	5.0	248	364.0	206
96	6.5	272	338.5	228

(a) Total amount of DL-methionine 1-C-14 added = 617 ng/mg VS.
(b) 5.0 μc of DL-methionine 1-C-14 added to 15 ml of sludge.
(c) Activity of DL-methionine 1-C-14 added = 46.7 ng/nc.
(d) 1.0 μl of sludge = 25.275 μg VS.

Table A-12

Gas Production and Composition, DL-Methionine 1-C-14 Added

Contact Time	Radioactive Gas Production			Percent of Total Applied Activity Appearing as Gas	Amino Acid Converted to Gas $\frac{ng}{mg\ VS}$			Rate of Amino Acid Converted to Gas During Increment $\frac{ng}{mg\ VS\ hr}$		
Hours (1)	CH_4 nc (2)	CO_2 nc (3)	Total nc (4)	(5)	CH_4 (6)	CO_2 (7)	Total (8)	CH_4 (9)	CO_2 (10)	Total (11)
0	0	0	0	0	0	0	0	0	0	0
0.0833	5.72	7.30	13.02	0.26	0.705	0.902	1.62	8.46	10.824	19.44
0.1667	6.85	22.4	29.25	0.58	0.845	2.71	3.61	1.68	21.696	23.88
0.50	4.03	70.8	74.83	1.50	0.500	8.73	9.20	-1.035	18.06	16.77
1	7.77	192	199.8	4.00	0.957	24.15	24.6	0.914	30.84	30.8
3	25.5	887	912.5	18.2	3.15	109.3	112.5	1.096	42.56	43.95
6	43.2	1885	1928	38.5	5.28	232	237	0.71	40.90	41.50
12	74.8	1900	1975	39.5	9.20	234	243	0.653	0.333	1.00
25.5	104	1880	1984	39.3	12.80	232	245	0.29	-0.16	0.16
48.5	125.3	1888	2013	40.2	14.50	233	248	0.074	0.04	0.13
96	183.7	2020	2204	44.0	22.55	249	272	0.170	0.337	0.505

Table A-13

L-Tyrosine 1-C-14 Uptake and Utilization

Contact Time Hours	Free Amino Acid in the Supernatant (35°C) ng/mg VS (1)	Amino Acid Converted to Gas (35°C) ng/mg VS (2)	Amino Acid Taken up by the Sludge (35°C) ng/mg VS (3)	Non-biological Uptake (4°C) ng/mg VS (4)
0	154.4	0	0	0
0.0833	119.6	2.8	32.0	28.7
0.1667	104.2	3.3	46.9	16.4
0.50	36.0	30.6	87.8	11.4
1	5.76	58.6	90.0	17.9
3	3.41	72.3	78.7	17.0
6	3.52	75.4	75.5	-
12	3.44	62.8	88.2	15.8
24	3.54	73.2	77.7	17.9
48	3.07	81.5	69.8	46.4
96	2.88	79.2	72.3	53.4

(a) Total amount of L-tyrosine 1-C-14 added = 154.4 ng/mg VS.
(b) 5.0 μc of L-tyrosine 1-C-14 added to 10 ml of sludge.
(c) Activity of L-tyrosine 1-C-14 added = 6.82 ng/nc.
(d) 1.0 μl of sludge = 22.080 μg VS.

Table A-14

Gas Production and Composition, L-Tyrosine 1-C-14 Added

Contact Time	Radioactive Gas Production			Percent of Total Applied Activity Appearing as Gas	Amino Acid Converted to Gas $\frac{ng}{mg\ VS}$			Rate of Amino Acid Converted to Gas During Increment $\frac{ng}{mg\ VS\ hr}$		
Hours (1)	CH_4 nc (2)	CO_2 nc (3)	Total nc (4)	(5)	CH_4 (6)	CO_2 (7)	Total (8)	CH_4 (9)	CO_2 (10)	Total (11)
0	0	0	0	0	0	0	0	0	0	0
0.0833	6.90	84.5	91.4	1.83	0.213	2.605	2.82	2.556	31.26	33.84
0.1667	8.34	100.0	108.3	2.16	0.25	3.09	3.34	0.528	5.82	6.24
0.50	29.8	964	994	19.9	0.92	29.75	30.65	1.989	79.98	81.93
1	37.4	1875	1912	38.2	1.167	57.4	58.6	0.494	55.30	55.90
3	62.2	2280	2342	46.8	1.916	70.4	72.3	0.375	6.50	6.85
6	115.2	2330	2445	48.8	3.56	71.9	75.4	0.548	0.50	1.04
12	93.6	1942	2036	40.7	2.89	59.9	62.8	-0.11	-2.00	-2.11
24	98.1	2275	2373	47.4	3.03	70.2	73.2	0.012	0.858	0.866
48	126.0	2515	2641	52.8	3.89	77.6	81.5	0.036	0.308	0.345
96	136.2	2430	2566	51.4	4.21	75.0	79.2	0.007	-0.054	-0.047

Table A-15

Summary, Maximum Gas Production Rates

	Methane Gas			Carbon Dioxide Gas			Methane Plus Carbon Dioxide Gas			
	$C^{14}H_4$ Only		$C^{14}H_4$ and CH_4 Gas from Tagged Amino Acid	$C^{14}O_2$ Only		$C^{14}O_2$ and CO_2 Gas from Tagged Amino Acid	$C^{14}H_4$ and $C^{14}O_2$ Only		$C^{14}H_4$, $C^{14}O_2$, CH_4, and CO_2 Gas from Tagged Amino Acid	
Amino Acid	ng of Amino Acid Converted to Gas/mg VS/hr	nl of Gas/mg VS/hr		ng of Amino Acid Converted to Gas/mg VS/hr	nl of Gas/mg VS/hr		ng of Amino Acid Converted to Gas/mg VS/hr	nl/mg VS/hr	µl/hr	µM Amino Acid/hr
	$\frac{ng}{mg\ VS\ hr}$ (1)	$\frac{nl}{mg\ VS\ hr}$ (2)	$\frac{\mu l}{hr}$ (3)	$\frac{ng}{mg\ VS\ hr}$ (4)	$\frac{nl}{mg\ VS\ hr}$ (5)	$\frac{\mu l}{hr}$ (6)	$\frac{ng}{mg\ VS\ hr}$ (7)	$\frac{nl}{mg\ VS/hr}$ (8)	$\frac{\mu l}{hr}$ (9)	$\frac{\mu l}{\mu M\ hr}$ (10)
Glycine	0.153	0.0514	0.985	1.705	0.573	1.10	1.740	0.585	1.12	9.34
Alanine	0.642	0.182	0.363	8.23	2.33	4.65	8.52	2.42	4.82	7.64
Leucine	0.325	0.0625	0.0728	7.72	1.485	1.73	7.98	1.536	1.79	9.04
Methionine	0.423	0.0716	0.568	2.125	0.359	2.85	2.19	0.370	2.94	1.88
Tyrosine	1.06	0.148	0.0788	33.2	4.62	2.46	34.0	4.73	2.52	13.33

(a) See Table A-16 for sample calculations.

Table A-16

Sample Calculations, Glycine 1-C-14

1) Column 10, Table A-6

Maximum rate of amino acid conversion to CO_2 gas = $\frac{13.12\ \text{ng}}{\text{mg VS hr}}$

2) Activity of glycine 1-C-14 = 9.00 ng/nc.

3) Specific activity of glycine 1-C-14 = 8.35 μc/μM.

4) Weight of only the radioactive amino acid molecules per nc of activity:

$$\frac{8.35\ \mu\text{c}}{\mu\text{M}} \times \frac{9.00\ \text{ng}}{\text{nc}} \times \frac{\mu\text{M}}{64\ \mu\text{c}} = \frac{1.17\ \text{ng}}{\text{nc}}$$

(See discussion in Chapter 7.)

5) Weight of $C^{14}O_2$ produced from only the radioactive glycine molecules:

$$\frac{\text{mol. wt. of } CO_2}{\text{mol. wt. of glycine}} \times \frac{1.17\ \text{ng}}{\text{nc}} = \frac{44\ (1.17)}{75.068} = \frac{0.686\ \text{ng}}{\text{nc}}$$

6) Maximum rate of $C^{14}O_2$ gas production, in terms of ng of $C^{14}O_2$ produced/mg VS hr.

$$\frac{13.12\ \text{ng}}{\text{mg VS hr}} \times \frac{\text{nc}}{9.00\ \text{ng}} \times \frac{0.686\ \text{ng}}{\text{nc}} = \frac{1.00\ \text{ng of } C^{14}O_2 \text{ gas}}{\text{mg VS hr}}$$

Table A-16 (Continued)

7) $C^{14}H_4$ is produced from $C^{14}O_2$.

8) Column 4, Table A-15

Maximum rate of conversion of only the radioactive glycine molecules to $C^{14}O_2$ gas, represented in terms of ng of glycine converted to gas/mg VS hr.

$$\frac{1.00\ \text{ng}}{\text{mg VS hr}} \times \frac{\text{mol. wt. glycine}}{\text{mol. wt. } CO_2} = 1.00\ \frac{(75.068)}{44} =$$

$$1.705\ \frac{\text{ng of glycine converted to } C^{14}O_2 \text{ gas}}{\text{mg VS hr}}$$

9) Column 5, Table A-15

Maximum rate of conversion of only the radioactive glycine molecules to $C^{14}O_2$ gas, represented in terms of nl of glycine converted to $C^{14}O_2$ gas/mg VS hr.

$$1.705\ \frac{\text{ng}}{\text{mg VS hr}} \times \frac{25.22}{\text{mol. wt. of glycine}} = 1.705\ \frac{(25.22)}{75.068} =$$

$$0.573\ \frac{\text{nl of glycine converted to } C^{14}O_2 \text{ gas}}{\text{mg VS hr}}$$

Table A-16 (Continued)

Also

$$1.00\ \frac{\text{ng of } C^{14}O_2 \text{ gas}}{\text{mg VS hr}} \times \frac{25.22}{\text{mol. wt. of } CO_2} =$$

$$1.00\ \frac{(25.22)}{44} = 0.573\ \frac{\text{nl of } C^{14}O_2 \text{ gas}}{\text{mg VS hr}}$$

At 1 atmosphere pressure and 35°C, 1 mole gas occupies 25.22 liters.

10) Column 6, Table A-15

Maximum rate of conversion of glycine 1-C-14 to CO_2 and $C^{14}O_2$ gas, represented in terms of µl of gas/hr.

$$0.573\ \frac{\text{nl}}{\text{mg VS hr}} \times \frac{249.5 \text{ mg VS in sample}}{1} \times \frac{\mu\text{l}}{1000 \text{ nl}} \times$$

$$0.009\ \frac{\mu\text{g}}{\text{nc}} \times \frac{\text{nc}}{0.00117\ \mu\text{g}} = 1.10\ \frac{\mu\text{l}}{\text{hr}}$$

Also from Column 3, Table A-6

$$\frac{30.43 \text{ nc}}{5 \text{ min}} \times \frac{60 \text{ min}}{\text{hr}} \times \frac{0.009\ \mu\text{g}}{\text{nc}} \times \frac{\mu\text{M}}{75.068\ \mu\text{g}} \times \frac{25.22\ \mu\text{l}}{\mu\text{M}} = \frac{1.10\ \mu\text{l}}{\text{hr}}$$

Table A-16 (Continued)

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11) Column 10, Table A-15

$$\text{Column 9} \; \frac{\text{mol. wt. of glycine}}{\mu\text{g of glycine added to the sludge}} =$$

$$1.12 \frac{\mu\text{l}}{\text{hr}} \times \frac{10^{3}\,\text{ng}}{\mu\text{g}} \times \frac{75.068\ \mu\text{g}}{\mu\text{M}} \times \frac{\text{mg VS}}{36.1\ \text{ng}} \times \frac{1}{249.5\ \text{mg VS}} = \frac{9.34\ \mu\text{l}}{\mu\text{M hr}}$$

12) ng = nanograms = 10^{-9} grams.

13) nl = nanoliters = 10^{-9} liters.

14) µl = microliters = 10^{-6} liters.

15) Activity calculations for L-alanine 1-C-14.

a) Total weight of alanine per nc of activity

$$\frac{\text{nM}}{8.00\ \text{nc}} \times \frac{89.095\ \text{ng}}{\text{nM}} = 11.2\ \frac{\text{ng}}{\text{nc}}$$

b) Weight of radioactive alanine only per nc of activity

$$8.00\ \frac{\text{nc}}{\text{nM}} \times \frac{\text{nM}}{64\ \text{nc}} \times 11.2\ \frac{\text{ng}}{\text{nc}} = 1.4\ \frac{\text{ng}}{\text{nc}}$$

Table A-16 (Continued)

c) Total weight of L-alanine 1-C-14 added to the sludge

$$\frac{5\ \mu c}{15\ \text{ml sludge}} \times 11.2\ \frac{ng}{nc} \times \frac{\text{ml sludge}}{16.575\ \text{mg VS}} \times \frac{10^{3}\ nc}{\mu c} =$$

$$226\ \frac{\text{ng of alanine}}{\text{mg VS}}$$

Table A-17

Maximum Total Uptake Rates
(From Observed Data)

Amino Acid	Maximum Total Uptake Rate at 35°C		Total Uptake Rate at 35°C During the First 5 Minutes	For Data See Table No.
	Rate in ng of Amino Acid Taken up per mg VS per hr $\frac{ng}{mg\ VS\ hr}$	Time Interval Minutes	Rate in ng of Amino Acid Taken up per mg VS per hr $\frac{ng}{mg\ VS\ hr}$	
Glycine	195.6	0 to 5	195.6	A-5
Alanine	493.2	0 to 5	493.2	A-7
Leucine	42.0	10 to 30	22.8	A-9
Methionine	232.8	0 to 5	232.8	A-11
Tyrosine	384.0	0 to 5	384.0	A-13

Table A-18

Amino Acid Chromatograms Exposed to X-Ray Film (Radioautographs), 35°C Biological Radioactive Studies

Contact Time	Glycine		Alanine		Leucine		Methionine			Tyrosine		
	Average Counts per Minute		Average Counts per Minute		Average Counts per Minute		Average Counts per Minute			Average Counts per Minute		
Hours	at origin cpm	at amino acid spot cpm	at origin cpm	at amino acid spot cpm	at origin cpm	at amino acid spot cpm	at origin cpm	at amino acid spot cpm	at secondary amino acid spot cpm	at origin cpm	at amino acid spot cpm	at secondary amino acid spot cpm
0 Standard	2.50	56.8	1.85	185.1	5.4	221.1	6.82	127.9	3.93	2.82	167	65.5
0.0833	1.00	28.3	2.2	173.1	4.23	197.7	7.65	118.2	4.62	2.17	151	64.4
0.1667	0.93	16.9	2.2	148.8	4.63	170.8	7.69	118.4	3.07	1.63	133	47.1
0.50	0.90	1.80	2.15	99.4	2.20	124.9	6.32	119.1	2.97	1.53	44.6	13.2
1	1.57	1.80	0.60	5.67	0.23	9.1	5.73	108.2	3.17	0.93	2.90	0.33
3	0.93	0.60	0.85	2.03	0.27	0.57	3.22	35.1	1.13	1.46	1.43	0.20
6	0.90	0.50	0.93	0.47	0.43	1.66	0.90	0.50	0.30	0.51	1.07	0.00
12	0.97	0.62	0.78	0.22	0.56	1.36	1.15	1.26	0.40	0.84	1.10	0.13
24	0.50	0.55	0.29	0.65	0.77	2.33	0.33	0.00	0.20	0.49	0.63	0.50
48	0.70	0.55	0.31	0.10	0.50	0.33	0.60	0.33	0.30	0.67	0.73	0.13
96	0.63	0.51	0.60	0.45	0.23	1.36	0.47	0.20	0.47	0.58	0.40	0.30

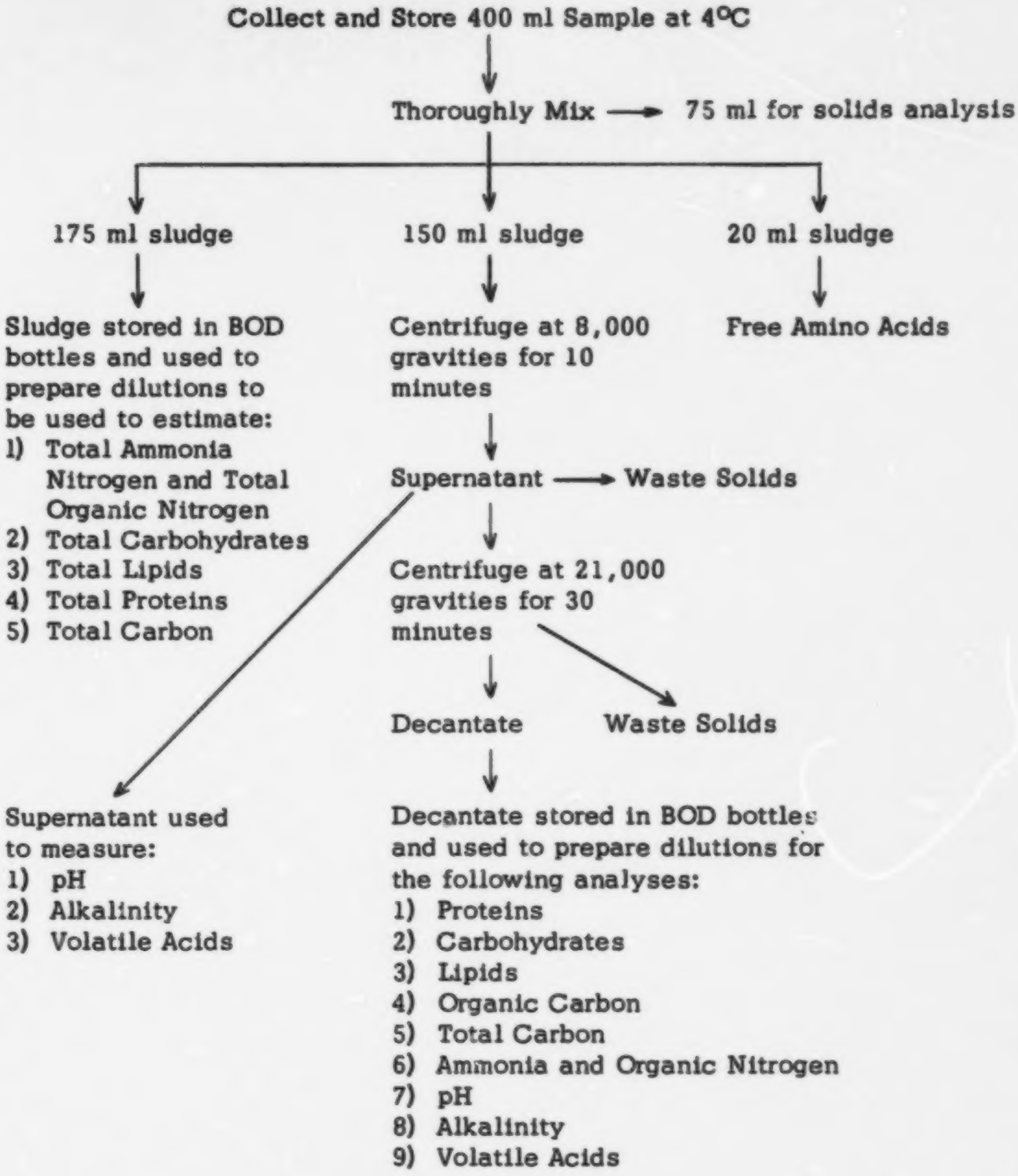
(a) 15 µl of solution applied to each chromatogram.

APPENDIX B

B-1 SAMPLE PREPARATION (164)

The collected sample was placed in a stoppered bottle and stored in a refrigerator until analysis. Prior to testing, the sample was thoroughly mixed by vigorous manual shaking of the bottle.

A diagram of the sample preparation is shown below.



Collect and Store 400 ml Sample at 4°C

Thoroughly Mix → 75 ml for solids analysis

175 ml sludge

Sludge stored in BOD bottles and used to prepare dilutions to be used to estimate:
1) Total Ammonia Nitrogen and Total Organic Nitrogen
2) Total Carbohydrates
3) Total Lipids
4) Total Proteins
5) Total Carbon

150 ml sludge

Centrifuge at 8,000 gravities for 10 minutes

Supernatant → Waste Solids

Centrifuge at 21,000 gravities for 30 minutes

Decantate

Waste Solids

Decantate stored in BOD bottles and used to prepare dilutions for the following analyses:
1) Proteins
2) Carbohydrates
3) Lipids
4) Organic Carbon
5) Total Carbon
6) Ammonia and Organic Nitrogen
7) pH
8) Alkalinity
9) Volatile Acids

Supernatant used to measure:
1) pH
2) Alkalinity
3) Volatile Acids

20 ml sludge

Free Amino Acids

The various dilutions required for each test were prepared with ammonia-free distilled water.

B-2 ALKALINITY AND VOLATILE ACIDS (164)

The concentration of alkalinity and volatile acids was evaluated on a 50-ml portion of the supernatant that had been separated from the solids by centrifuging at 8,000 gravities for ten minutes. A Beckman Model N pH meter with glass and calomel electrodes was used for these analyses.

The concentration of alkalinity was estimated by titrating a 50-ml sample with 0.1 N sulfuric acid to an endpoint of pH 4.5 (i.e., the average pH for color change). The results were expressed as milligrams per liter of calcium carbonate.

Volatile acids were determined on the same sample by direct titration (38). The sample was acidified to pH 3.3 with sulfuric acid, boiled gently for three minutes on a hot-plate, cooled to room temperature, and the pH of the sample was raised to 4. The volume of 0.1 N sodium hydroxide required to raise the pH from 4 to 7 was recorded and used as a measure of the volatile acids content. The results were expressed as milligrams per liter of acetic acid.

Calculations

Alkalinity, mg/l calcium carbonate = $(V_1)(100)$

Volatile acids, mg/l acetic acid = $(V_2)(120)$

where V_1 = volume of 0.1 N sulfuric acid required to lower the pH of sample to 4.5

and V_2 = volume of 0.1 N sodium hydroxide required to raise the pH of sample from 4.0 to 7.0

B-3 CARBOHYDRATES (ANTHRONE REACTION) (164)

Carbohydrates are organic compounds that are polyhydroxyaldehydes or polyhydroxyketones, and their derivatives (159). These compounds are generally white solids, sparingly soluble in organic liquids, and, except for certain polysaccharides, soluble in water. Carbohydrates contain carbon, as well as hydrogen and oxygen, in the same ratio as these elements combine in water (126).

A great many qualitative and quantitative tests for carbohydrates have been developed. Most of these tests are for a specific carbohydrate or class of carbohydrates. The analytical procedures developed by Fehling, Benedict, and Barford depend upon the reduction of cupric ions to cuprous oxide by reducing sugars (66). The Seliwanoff test involves the red color complex caused by the action of resoricinol and hydrochloric acid on ketoses (159). Starch and glycogen are detected by an iodine reaction (66).

The most generally applicable test for carbohydrates in wastewater is the Anthrone Reaction (59). This test was introduced in 1946 (42) and later modified (108). When anthrone is mixed with concentrated sulfuric acid and the carbohydrate, a blue or green color complex is formed. The intensity of the color developed is proportional to the concentration of carbohydrate in the sample. Anthrone does not react with organic acids, aldehydes, phenols, fats, terpenes, alkaloids, protein, acetate, and sugar alcohols (42,59,108). The only non-carbohydrate known to give this reaction is furfural (59).

Three precautionary measures must be observed to obtain reproducible results: namely,

1) The anthrone reagent must be ice-cold when added to the sample;

2) The sample must be ice-cold when the anthrone reagent is added; and

3) The boiling time for color development must be exactly 15 minutes.

A calibration curve should be prepared from a new carbohydrate standard solution each time the test is performed, unless there is no decrease from the last run. A calibration curve is prepared by running three tubes containing 0.25 mg of glucose and three tubes containing 2.5 ml distilled water as a blank plus the anthrone reagent to make the reagent blank. The intensity of the color is proportional to the concentration of the carbohydrate in this range.

Reagents

Anthrone Reagent--Dissolve 0.2 g of anthrone in 100 ml of 95 percent sulfuric acid. Store until cold in refrigerator. Do not prepare this reagent more than six hours prior to using.

95% Sulfuric Acid--Add 950 ml concentrated sulfuric acid to 50 ml distilled water.

Standard Glucose Solution--100 mg/l glucose (2.5 ml = 0.25 mg of glucose).

Procedure

1) Add 2.5 ml of sample or sample diluted to 2.5 ml into a 15 x 75 mm test

tube (e.g., unknown sample).

2) Add 2.5 ml of known concentrations of glucose into a test tube (e.g., standard sample).

3) Add 2.5 ml of distilled water to test tubes for blanks (e.g., reagent blank of standard sample).

4) Place all tubes in ice water bath until they are cold.

5) With tubes remaining in ice water bath, rapidly add 10 ml of anthrone reagent to each tube with a syringe and mix vigorously.

6) Cap each tube with a glass marble and place in a boiling water bath for exactly 15 minutes.

7) Remove all tubes from the boiling water bath and place into a cold water bath.

8) Mix contents of test tube.

9) Centrifuge sample if necessary to remove organic debris.

10) For more consistent and reproducible results, allow to stand at least 24 hours at 4°C before step 12.

11) Mix again before step 12.

12) Measure optical density at 620 mμ with spectrophotometer.

B-4 PROTEIN DETERMINATION (164)

An accurate estimate of the concentration of protein in sludge is difficult because no chemical reaction is known to be specific for proteins. Therefore, alternate procedures must be employed: namely, extraction of the proteins in a purified form or extraction of the interfering substances. There are methods available for extracting and purifying proteins that have been used on plant and animal tissues and bacterial cells that should be applicable to sludge (45). However, these methods are time-consuming and are not generally applicable to routine protein determinations.

However, many quantitative tests for estimating the amount of protein are available. These comparative techniques involve:

1) An estimate of the organic nitrogen concentration of the samples, or

2) A reaction of a specific amino acid or functional group generally associated with proteins.

The Kjeldahl analysis of the concentration of organic nitrogen is used to indicate the protein content. The actual nitrogen content of simple proteins varies from 14 to 20 percent by weight; however, most conjugated proteins contain less nitrogen than the simple proteins. A generally accepted assumption is that nitrogen makes up 16.5 percent of the weight of most proteins. It has not been established that all organic nitrogen in sludges is associated with protein.

Chemical agents have been used to precipitate the proteins in order to separate these compounds from other nitrogenous materials. The organic nitrogen content of the precipitated protein is evaluated.

Trichloroacetic acid (commonly called TCA) can be used to remove the proteins. TCA will precipitate most proteins with the exceptions of the mucoids or glycoproteins (45). Amino acids or low molecular weight peptides are not removed by TCA (98). In certain biological fluids it is assumed that TCA will not precipitate materials other than proteins (98). However, there is no evidence that this assumption would be valid when TCA is added to a complex material sludge. Some precipitating agents, such as metaphosphoric acid, will partially hydrolyze the proteins causing a decrease in protein nitrogen and an increase in amino acid nitrogen; however, the use of TCA does not give rise to this error (36).

There are several colorimetric tests which depend upon the presence of a particular structure or amino acid in the protein. The Millon Reaction (66) is dependent upon the reaction between the phenolic group of the amino acid tyrosine and the reagents which contain nitrite and mercuric nitrate in a mixture of nitric and nitrous acids. Another typical example of this type reaction is the Hopkin-Cole reaction which is dependent upon the presence of tryptophan in the protein (66). The ninhydrin reaction is also used for protein analysis.

A promising method of protein determination in sludge is the Biuret reaction (59). A protein in an alkaline solution is mixed with a weak solution of copper sulfate resulting in the formation of a violet color. This reaction depends upon the presence of two or more peptide linkages. Ammonium ion will interfere with the Biuret test (59). The ammonium ion can be removed from sludge by filtering and washing the sludge prior to analysis. An unknown interfering substance was present in the sludges investigated. This substance was not water-soluble and reacted with the Biuret reagent by forming a brick-red color, similar to the reaction expected of reducing sugars. This brick-red color completely masked the violet color and could not be removed by centrifuging for one-half hour at 21,000 gravities.

The method used for the routine determination of proteins in this investigation involved the reaction of Folin Phenol Reagent with the tyrosine and tryptophan of the protein resulting in the formation of a blue-colored complex (85).

The advantages of this test include: (1) no apparent interfering substances were present in the sludges tested; (2) this determination is more sensitive than ninhydrin and 100 times more sensitive than the Biuret reaction (85); and (3) the analysis was very easy to perform.

The disadvantages of the Folin analysis are: (1) the amount of color developed varies with each protein; (2) the color is not proportional to the concentration of protein; therefore, a calibration curve over the full range of expected concentrations must be prepared; and (3) the results must be expressed in terms of the protein used for the calibration curve. Different results are found with each protein used as the standard.

Reagents

Folin A--Mix 20 g Na_2CO_3 with 4 g NaOH and dilute to 1 liter with distilled water. This reagent is stable for 2 to 3 months if it is not contaminated.

Folin B--Mix 100 mg sodium tartrate with 50 mg $CuSO_4$ in 10 ml distilled water. This reagent is stable for one week if both components are together. For stable solutions, dissolve 100 mg sodium tartrate in 5 ml H_2O (and store at 4°C) and dissolve 50 mg $CuSO_4$ in 5 ml H_2O. Mix 5 ml of each when ready to use. If made in this manner, the solution is stable.

Folin C--1 ml phenol reagent (Fisher Cat. No. SO-P-24) is mixed with 1.2 ml distilled water. Phenol reagent is stable for several months; however, after dilution with water, the reagent should be used within one hour. Mix just prior to reaction.

Folin Mixture--Mix 1 ml of Folin B to 49 ml of Folin A immediately before adding.

Procedure

1) All total sludge samples should be vigorously mixed before making any dilutions and prior to step 2.

2) The sample plus dilution water should total 1 ml and is added to a test tube.

3) Add 5 ml of Folin Mixture (A + B) to the sample, thoroughly mix, and let stand for 15 minutes at room temperature.

4) Add 0.5 ml of Folin C and mix immediately.

5) Let color develop for 30 minutes at room temperature. After 30 minutes the test tube contents may be centrifuged if necessary to remove organic debris.

6) Determine optical density at 700 mμ with spectrophotometer. Sample must be read on spectrophotometer within one hour after color development. Color begins to fade after one hour. All samples approach a common optical density.

Calibration

A calibration curve using a standard protein should be prepared. In this investigation Bovine Serum Albumin was used as the standard protein. A calibration curve utilizing Knox "Gelatin" was also prepared for comparison purposes. The curve should cover the range of 0 to 150 μg of protein in 1 ml volume.

A four-point calibration curve (0, 45, 75, and 150 μg) was prepared each time the protein determination was peformed.

B-5 LIPID DETERMINATION (164)

The name "lipid" is usually given to the material that is soluble in ether, chloroform, benzene, petroleum, carbon disulfide, and the other fat solvents. Lipids may be defined as those materials that are soluble in nonpolar solvents (159). This term includes the neutral fats, waxes, phospholipids, cerebrosides, aliphatic alcohols, glyceryl ethers, and the products of partial or complete hydrolysis of any of the above.

In Standard Methods (9), the term "grease" is used and is defined as that material which is extracted from an acidified sample by petroleum ether with a boiling point of 35° to 54°C (9).

The method for the extraction of lipids used in this investigation is a wet extraction procedure utilizing chloroform and methanol (84). According to Loehr (84):

> In this method, optimum lipid extraction occurs when chloroform and methanol are homogenized with the water in an acidified sample to form a monophasic solution. The resulting mixture is then diluted with additional water and chloroform to produce a biphasic system. The chloroform layer of the biphasic system contains the lipids and the water-methanol layer contains the non-lipids. A purified lipid extract is obtained when the chloroform layer is isolated.

The quantity of lipids is determined gravimetrically.

Reagents

Methanol--analytical reagent grade.

Chloroform--analytical reagent grade.

Hydrochloric acid (1 N).

Special Equipment

Waring Blender

250-ml capacity separatory funnels with a suction outlet and teflon stopcock.

Procedure

1) A sample containing approximately 50 ml of sludge is homogenized in a clean Waring Blender and then returned to the original container. The blender is then cleaned with chloroform and methanol prior to further use.

2) 20 ml of chloroform and 50 ml of methanol are added to 25 ml of the sample in the blender. Use the same graduate cylinder to measure the volume of sample, chloroform, and methanol so that none of the sample is left in the graduate.

3) Acidify the sample with H_2SO_4 to pH 4-4.5 and blend for one minute.

4) Add 30 ml of chloroform and blend for 30 seconds.

5) Add 25 ml of distilled water and homogenize for an additional 30 seconds. (The addition of the chloroform and water can be made without stopping the blender.)

6) The contents of the blender are filtered by vacuum with suction through a Buchner Funnel into the separatory funnel. The blender is rinsed with 15 ml of methanol which is added to the filter. After all of the liquid passes into the separatory funnel, the vacuum is stopped, and the Buchner Funnel is removed.

7) After the two solvent phases separate (about 5 minutes) the bottom chloroform layer is withdrawn into a tared 100 ml beaker. A small amount of chloroform is left in the funnel. The phases usually break within five minutes. When an interfacial emulsion remains, it is not withdrawn with the chloroform bottom layer.

8) 10 ml of chloroform are added to the separatory funnel, mixed, and then removed to the tared beaker after the phases break.

9) The solvent is evaporated from the tared beaker in a water bath at 80°C.

10) The tared beaker is dried in a 103°C oven for five minutes. The dried beaker is cooled for 30 minutes in a desiccator and weighed. It is necessary to run a reagent blank with distilled water.

The gain in weight of the tared beaker less the reagent blank is the weight of the lipids. The results are calculated and expressed in terms of mg/l.

B-6 FREE AMINO ACIDS (164)

Free amino acids are those amino acids that are in solution and do not include the amino acids which are connected by peptide linkages. Amino acids can be determined by separating and measuring each individual amino acid separately or by measuring all of the amino acids collectively by a common chemical reaction.

Separation and measurement of individual amino acids generally involves chromatography. Amino acids in sludge have been qualitatively and quantitatively evaluated by paper chromatographic techniques (115, 125). A common method for removing amino acids from a heterogeneous mixture of compounds is by extraction with ethyl alcohol (45). The alcohol soluble fraction contains a mixture of amino acids, glutathione, lipids, proteins, and other organic compounds (120). The lipids can be removed by extraction with ether, and the proteins can be precipitated with trichloroacetic acid (TCA). The remaining amino acids and peptides can be concentrated and analyzed by chromatographic techniques. It is usually necessary to concentrate the samples because of the low free amino acid concentrations present in sludges (115, 125). Prior to analysis by paper chromatography, the TCA and salts must be removed from the solution because these compounds alter the R_f factors of amino acids. The TCA and salts can be removed in one step by a Dowex-50 NH_4 desalting procedure, although this method will not give quantitative recovery of basic amino acids (166).

Chromatographic techniques are too tedious and time-consuming for use with sludges when only an estimate of the total free amino acids content of the sludge is necessary. Chromatographic techniques applied to free amino acids determination in sludge would also require a larger sample than a direct determination of total free amino acids.

The determination of total free amino acids used in this investigation is based on the reaction of these compounds with ninhydrin. Amino acids will react with ninhydrin to yield carbon dioxide, an aldehyde containing one less carbon than the amino acid, and a blue or purple color (159).

The ninhydrin reaction is not specific for amino acids, but it is widely used to provide a colorimetric determination of amino acids. Proteins, peptides, ammonia, and other organic compounds such as imino acids, amino alcohols, and primary amines also react with ninhydrin.

The technique used in these studies was intended for the quantitative analysis of concentrations of a single amino acid (85,139). However, this technique is applicable to mixtures of amino acids, provided that allowances are made for the slight variability of the color yields resulting from the ninhydrin reaction with the various amino acids and the presence of interfering compounds (139). All of the alpha-amino acids found in proteins give approximately equal color yields on a molar basis (139). Proline and hydroxyproline (imino acids) give a different color reaction (139). A mixture of alpha-amino acids will yield a color that is approximately equal to the color yield of an equal concentration (mole basis) of a single alpha-amino acid such as glycine (139).

The removal of interfering compounds from the sludge is necessary.

The sample preparation used in this investigation consisted of the following steps:

1) The free amino acids were separated from the bulk of the insoluble organic and inorganic compounds by centrifugation.

2) The decantate contained the free amino acids plus many other soluble compounds. The proteins were precipitated by the addition of TCA and removed by centrifugation.

3) The ammonia was removed by making the sample alkaline, followed by boiling and sparging the sample with acid-washed nitrogen gas (35).

4) The pH was adjusted to 7.0 for analysis by the ninhydrin reaction.

This technique yielded 82 percent recovery of glycine. The recovery of other amino acids was not evaluated.

A. Sample Preparation for Amino Acids Determination

Reagents

Sodium Hydroxide--15 N

25% Trichloroacetic Acid Solution (TCA)--Add 25 g of trichloroacetic acid to ammonia-free distilled water to make a total volume of 100 ml.

The following procedure was used to prepare the samples for the free amino acids analysis.

1) A 20 ml sample of the sludge was centrifuged for 5 minutes at 8,000 gravities. The decantate was retained.

2) The decantate was centrifuged for 30 minutes at 21,000 gravities. The decantate was retained.

3) 5 ml of 25 percent TCA was added to the decantate and the mixture was allowed to stand at least 15 minutes.

4) 4 ml of 15 N sodium hydroxide was added to the decantate. Any precipitate that formed was removed by centrifuging for 5 minutes at 4,000 gravities.

5) The decantate was transferred to a glass tube and was placed in a boiling water bath. Acid-washed (1 + 1 sulfuric acid) nitrogen gas was bubbled through the decantate during boiling to strip out NH_3. The sample was boiled for four minutes.

6) The sample was allowed to cool to room temperature. Any precipitate that formed was removed by centrifuging for five minutes at 8,000 gravities. If precipitate is not removed, then centrifuge at 11,000 gravities for five minutes.

7) The pH of the decantate was adjusted to 7.0 by adding 10 N sulfuric acid, dropwise.

8) The decantate was diluted to 20 ml with ammonia-free distilled water. If decantate volume is greater than 20 ml, then note volume for future calculations.

9) The decantate was stored in a refrigerator until the analysis for free amino acids was performed.

B. Free Amino Acids (Ninhydrin Reaction)

Reagents

Reagent A-1--Pierce Ninhydrin, 1 g. Peroxide Free Methyl

Cellosolve (also called 2-methoxyethanol or ethlene-glycol-monoethyl ether), 25 ml.

Reagent A-2--Mallinckrodt Stannous Chloride, 40 mg. 4 M pH 5.3 Acetate, 25 ml (use 4 M sodium acetate solution and adjust pH to 5.3 with glacial acetic acid).

Immediately prior to usage mix reagents A-1 and A-2 together. This makes 50 ml of reagent. Reagents A-1 and A-2 are made just prior to usage.

Procedure

1) If prepared sample from step A9 shows any trace of precipitate, then centrifuge at 21,000 gravities for 15 minutes.

2) Use 0.5 ml of prepared sample from step 1 above. Make dilutions so that sample plus dilution water totals 0.5 ml. Volume of standard glycine solutions for calibration curve should also total 0.5 ml after dilutions are made.

3) Prepare reagents A-1 and A-2.

4) Add 2 ml of reagent to samples and standards.

5) Place marble on tube and boil in water bath for 15 minutes.

6) Add 1 to 5 ml of 2-propanol (isopropanol) to each sample.

7) Any precipitate that forms can be removed by centrifuging for approximately 3 minutes at 4,000 gravities.

Calibration

Use standard solutions of glycine, 1/2 ml solution = 0.20 and 0.40 micromoles glycine and reagent blank.

B-7 TOTAL CARBON (164)

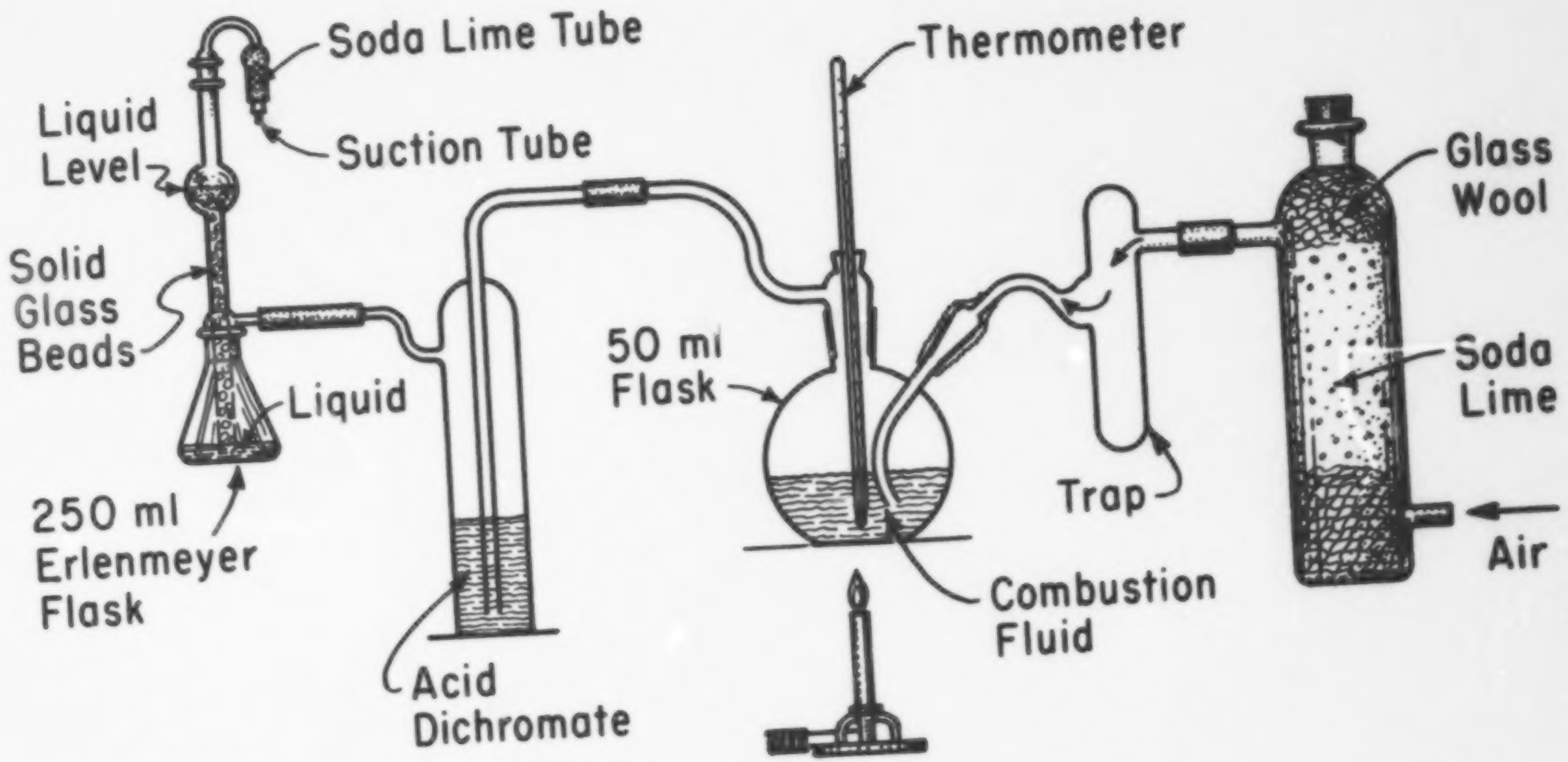
Total carbon was measured by a wet oxidation method using Van Slyke-Folch reagent as the oxidizing reagent (155,156). Van Slyke-Folch reagent is considered to closely approach an ideal universal reagent for measuring carbon. This technique gives reliable results even with volatile compounds such as ethanol or acetone (109).

Organic compounds are oxidized quantitatively to carbon dioxide if heated with the oxidizing agent. Carbon dioxide in carbonates is also liberated as carbon dioxide. There is no interference from nitrogen, sulfur, halogens, or alkali metals.

The carbon dioxide that is generated could be determined gravimetrically, volumetrically by gasometric techniques. In this procedure, the CO_2 is absorbed in an excess of carbonate-free sodium hydroxide. The excess sodium hydroxide that remains after CO_2 absorption is determined by titration. This method permits the isolation of carbon in a form suitable for radiotracer measurements.

The apparatus used is shown in Fig. B-7-1. The sample and combustion fluid are placed in the combustion flask. Carbon-dioxide free air is used to sweep the carbon dioxide from the heated combustion flask through a bubbler into a bead tower charged with sodium hydroxide.

The bubbler gives a check on the rate of flow of the air stream and contains acid dichromate solution which is used to absorb volatile oxides of nitrogen and sulphur. The apparatus can be operated under a vacuum or pressure.


Soda Lime Tube
Suction Tube
Liquid Level
Solid Glass Beads
Liquid
250 ml Erlenmeyer Flask
Acid Dichromate
Thermometer
50 ml Flask
Combustion Fluid
Trap
Glass Wool
Soda Lime
Air

APPARATUS FOR TOTAL CARBON ANALYSIS

FIG. B-7-1

The carbon dioxide that is swept from the combustion flask into the bead tower is absorbed by the carbonate-free sodium hydroxide.

$$2\ NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

In order to prevent the formation of an appreciable amount of bicarbonate, the sodium hydroxide should be added in excess of that estimated stoichiometrically from the above equation. Twice the theoretical amount has proved to be satisfactory (109).

An excess of barium chloride is added to precipitate the carbonate.

$$Na_2CO_3 + BaCl_2 \longrightarrow BaCO_3 + 2\ NaCl$$

The excess sodium hydroxide can be determined by titration with hydrochloric acid to the thymolphthalein end point since barium carbonate does not react with acids at this pH range (109).

Reagents

Potassium Iodate--powdered reagent grade potassium iodate. Weigh out 1.2 g aliquots and store in stoppered test tubes until required.

Sodium Hydroxide (1 N)

Van Slyke-Folch Combustion Fluid--Place 25 g chromium trioxide, 5 g powdered potassium iodate, and 167 ml of 85 percent phosphoric acid in a one-liter glass-stoppered flask. Add 333 ml of fuming sulfuric acid

(20 percent SO_3). Heat the mixture to 150°C with occasional stirring. Cool with a beaker inverted over the mouth of the flask. When cool, insert the stopper and replace the inverted beaker over the stopper to keep dust from collecting on the mouth of the flask.

Carbon Dioxide Free Distilled Water--Boil distilled water for 15 to 20 minutes. Store in a glass bottle fitted with a soda-lime or ascarite tube. Kjeldahl burners and flasks are very convenient for boiling the water. After boiling, stoppers fitted with soda-lime tubes are placed on the flasks while the water cools.

Barium Chloride (1 M)--Dissolve 104 g of barium chloride (or 122 g of $BaCl_2 \cdot 2H_2O$) in carbon dioxide free water and dilute to 500 ml. Neutralize to the thymolphthalein end point with 1 N sodium hydroxide before dilution.

Hydrochloric Acid (1 N)--Add 84 ml of concentrated HCl to sufficient carbon dioxide free water to give 1 liter. Standardize against 1 N sodium hydroxide.

Thymolphthalein Indicator--Dissolve 100 mg of thymolphthalein powder in 100 ml of 80 percent ethanol.

Procedure

1) Add 10 ml of 1 N NaOH to the bead column. Dilute with 20 ml of carbon dioxide free distilled water. With this volume of fluid in the bead column, the bulb of the column is filled one-half full when in operation. The NaOH must be accurately measured with a pipette.

2) Add 1.2 g of powdered KIO_3 to the combustion flask. Use a funnel when transferring the KIO_3 from the test tube to the flask to avoid getting KIO_3 on the mouth of the combustion flask.

3) Pipette 4.0 ml of the solution to be analyzed into the combustion flask. (Not more than 80 mg of carbon should be added to the flask. With sludge, it is usually necessary to use a diluted sample.)

4) Lubricate joints on the combustion flask (including the thermometer) with syrupy phosphoric acid and connect combustion flask to absorption train.

5) Add 20 ml of combustion fluid to the combustion flask through the air inlet with a syringe. Connect trap as quickly as possible. Start flushing with carbon dioxide free air immediately. The oxidation of the organic materials generates heat which will cause an increase in pressure and force the sample and combustion fluid up into the trap with the resultant loss of the sample. Flushing with carbon dioxide free air will prevent this buildup of pressure.

6) Heat the combustion flask to 230°-250°C with a gas burner. When this temperature is reached, continue to pass a slow stream of CO_2 free air (2-4 bubbles per second) through the combustion flask. Hold the temperature at 230°-250°C for ten minutes.

7) After ten minutes turn off the heat. Increase the air stream and flush the system for five minutes.

8) After flushing the system, remove the bead column from the rest of the train.

9) Wash the NaOH from the bead tower into the flask with carbon dioxide free water. Use 25 ml of water.

10) Transfer the NaOH solution to the flask to be used for titration.

11) Add 10 ml of 1 M barium chloride solution to the flask.

12) Add five drops of thymolphthalein solution.

13) Titrate with 0.5 N hydrochloric acid. (Add HCl until the blue color just disappears.)

14) Calculate the amount of carbon present.

$$\frac{\text{(Titer of blank - titer of sample)}}{2}\text{(normality of HCl)} = \text{mM of } CO_2$$

Titer of blank = ml of HCl that react with NaOH

Titer of sample = ml of HCl that react with NaOH

B-8 MANOMETRIC METHODS

Manometric methods are the oldest and best established means for determining oxygen uptake (93) or gas production. The Warburg respirometer was used for gas production measurements since it provides a reliable method for measuring gas production at the short time intervals needed for plotting. In addition, this technique permits a large number of samples to be run at the same time under the same environmental conditions.

An 18 flask circular Precision Warburg respirometer as described by Lardy (81) was used in the gas production and amino acid uptake studies. The water bath temperature was maintained at 35.0°C ± 0.05°C for all the uptake studies. The flask size was 125 ml with three serum cap openings and a center well for the absorption of carbon dioxide in potassium hydroxide, if desired. The flask shaking rate was 60 oscillations per minute. In these experiments 15 ml of unaltered anaerobic digested sludge from the laboratory in 6-liter digesters plus 5 ml of ammonia-free distilled water for a total volume of 20 ml was used. Radioactive amino acid uptake studies used 15 ml of sludge and 100 µl of the radioactive amino acid. In experiments of amino acid uptake, a 1-ml volume of amino acid was added for a total volume of 21 ml. Flask constants were computed for each of the Warburg flasks based on 15, 20, and 21 ml liquid volumes. Since the numerical value of the flask constant is a function of the type of gas, gas chromatography was used to determine the type and quantity of gas in the Warburg flasks. An average value for the flask constant was then computed. It should be noted that in this environment, the quantity of each gas present and its percentage varies with time; hence, the flask constant is variable.

Krebs solution was used for the manometer indicating fluid (153). Fluid was injected and removed from the manometers through a serum cap opening at the bottom of the manometer reservoir. In addition, this method facilitated adjustments in fluid quantity, when necessary, in the anaerobic gas production studies.

Glass joints were lubricated with "Dow Corning Silicone Lubricant" prior to use. The flasks were cleaned by boiling twice with Alconox detergent and rinsing six times with distilled water.

Replacing the atmosphere of air in the empty Warburg flasks prior to the experiment was performed by the following sequence:

1) Fill up manometers with Krebs fluid.

2) Flush with helium gas through a number 23 needle inserted in the lower serum cap of the Warburg flask.

3) Exhaust the helium gas through the manometer tube opening on the upper right column.

4) Flush with helium gas for 15 minutes. Analyze gas content by withdrawing 1 ml of gas with a gas-tight syringe and inject into gas chromatograph. Five percent of air by volume was considered the maximum allowable air concentration.

5) Since sludge will not pass through a needle as large as number 12 and flushing with helium while the sludge was in the Warburg flasks caused liquid carry-over to the Krebs fluid, an alternative method was

used as follows:

a) The inoculum was drawn into a nalgene pipette with 1/2 inch of the tip removed.

b) Removal of a serum cap on the Warburg flask followed by rapid injection of the inoculum.

c) Replacement of the serum cap resulted in a minor amount of air entry.

Since the liquid inoculum displaced the flask atmosphere, there was a large change in the manometer liquid level. After attaching a serum cap to the manometer outlet with a number 25 needle inserted, it was possible to force the displaced gas out of the system whenever a liquid volume was put in through the serum caps or serum cap openings. Since a positive pressure exists, no air entry should occur. This serum cap on the manometer was also utilized for the removal of gas with a gas-tight syringe that is produced by the organisms in the Warburg flask.

6) The Warburg flask was then flushed with gas from the 6-liter digesters. This gas contained about 65 percent methane and 35 percent carbon dioxide. Carry-over of liquid does not occur under these conditions.

7) The Warburg flask and manometer were placed in the 35°C water bath. Temperature increase caused gas expansion. This gas was released through the manometer serum cap. The Warburg system was then ready for anaerobic studies on wastewater sludge.

B-9 UPTAKE OF AMINO ACIDS

The uptake rate was measured by adding 5 μc of amino acid to 15 ml of digesting sludge. The actual weight added varied considerably among amino acids because of the variance in specific activity of the amino acid solutions. The actual weights are presented in the text. The biological and non-biological uptake after 5, 10, 30 minutes and 1, 3, 6, 12, 24, 48, and 96 hours were determined.

The method of sample preparation for non-biological uptake was as follows. A sample of digesting sludge was cooled to 4°C. After the sludge had been at 4°C for at least 24 hours, 67 μl of tagged amino acid was added to 10 ml of sludge while in the 4°C room. At the proper time, 3 ml of 25 percent trichloroacetic acid was added, then the sample was centrifuged at 21,000 gravities (both steps done at 4°C). The supernatant was retained for counting.

In order to count the supernatant, a 10-μl volume of the supernatant was added to a 54-mm diameter circular disk of Whatman Number 1 filter paper in such a manner as to cover a circle with a 1/2 inch diameter. A quantitative measure was made possible by use of a calibration curve prepared with the tagged amino acid and the supernatant from the sludge being tested (zero non-biological uptake).

Total anaerobic uptake was determined at 35°C in Warburg flasks. The biological activity results in a portion of the amino acid being converted to carbon dioxide, which is subsequently reduced to methane, while the remainder is either associated with bacterial solids as a free amino acid in solution or some breakdown product of the amino acid. The difference between total uptake and non-biological uptake is the biological uptake. One hundred microliters of tagged amino acid are added to the 15 ml of wastewater sludge in an anaerobic environment in the Warburg flasks through serum caps. At time t, the biological activity is stopped with 5 ml of trichloroacetic acid and the sludge sample centrifuged as soon as possible thereafter at 21,000 gravities. A portion of the gas produced was withdrawn with a gas-tight syringe, separated by gas chromatography and transferred to an ionization chamber for subsequent measurement in the gaseous phase. The supernatant remaining after centrifugation at 21,000 gravities was added to Whatman Number 1 filter paper for measurement as discussed previously.

The gas produced (i.e., radioactive and non-radioactive carbon dioxide and methane) is measured with the Warburg manometer at various time increments, depending on the rate of gas production. Data is measured in terms of a pressure difference and converted to microliters of gas (153). Data is collected from time, zero, the injection of the 100 µl radioactive amino acid sample to time t, when the biological activity is stopped. (Typical data are plotted in Figs. 6-2 through 6-6.)

A 10-µl volume of supernatant from the total uptake studies was also added to sheets of Whatman Number 1 filter paper for paper chromatography separation. Paper chromatograms were run to determine the identity of the radioactive compounds resulting from the applied tagged amino acid. Since amino acids can break down to form various keto-acids, hydroxy acids, fatty acids, alcohols, and amines, paper chromatography followed by radioautography can facilitate the identification of these groups of compounds. A quantitative measure is then obtained by counting the radioactivity on the paper.

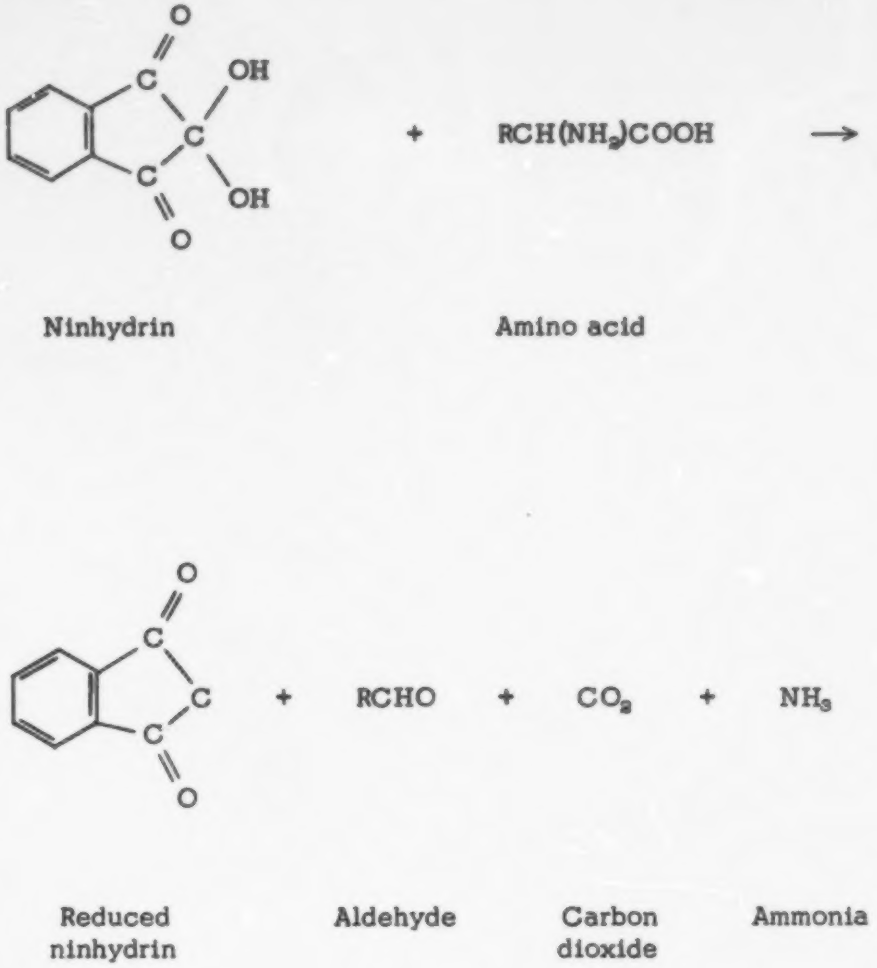
Colorimetric methods are not practical because of the lack of sensitivity and the production of adverse products (e.g., ninhydrin reacts with an amino acid to release carbon dioxide which would be radioactive in these studies).

B-10 CHROMATOGRAPHIC TECHNIQUES

Paper chromatography was used solely for the separation of very small amounts of amino acids and other organic compounds. Only radioactive solutions were separated by paper chromatography. The concentrations of these compounds were less than the detectable limits by colorimetric methods. Therefore, to identify the compounds separated on paper chromatograms, radioautographs were used. After identification of the compound, its concentration was determined by measuring the activity of the spot on the paper with a Sharp's Lowbeta radioactivity counting apparatus (8).

Samples analyzed by paper chromatography were applied on 0.140-inch diameter areas on Whatman Number 1 filter paper. The amino acids, if present, were separated by their different rates of travel in a descending solvent system. After traversing the chromatogram, the solvent is evaporated from the filter paper. Ordinarily, the color reagent, ninhydrin, is used to reveal the location of amino acids on the filter paper. As shown by Meister (103) and Kleiner (78) and others, most amino acids react with ninhydrin to yield carbon dioxide, ammonia, and the amino acid aldehyde according to the reaction shown on the following page.

Since the carboxyl carbon is labeled in the studies contained herein, it would not be desirable to produce carbon dioxide under such uncontrolled conditions. Therefore, the chromatograms are not developed but instead are exposed to Kodak Type No Screen X-ray film. The developed X-ray film shows the location of the radioactivity on the paper chromatogram


OH
OH
+
RCH(NH$_2$)COOH
Ninhydrin
Amino acid
+
RCHO
+
CO$_2$
+
NH$_3$
Reduced ninhydrin
Aldehyde
Carbon dioxide
Ammonia

and subsequent counting of the paper chromatogram yields its activity. The method of exposure of the paper chromatograms to the X-ray film consisted of placing the paper chromatogram and a sheet of X-ray film face-to-face in a light-tight X-ray film cassette for at least three weeks. The cassette was loaded and the film developed under safelight conditions.

A 10-µl syringe was used for the solution application and the solution applied in fractions of a microliter in order to keep the liquid within the 0.140-inch diameter circle. This was necessary since all solutions diffuse as they move, the eventual area being proportional (within limits) to the concentration (28). With radioactive solutions, the smaller the spot area, the more dense is the image on the X-ray film. To accelerate the rate of drying of the solution applied to the paper, a hot air stream from a hair dryer was directed across the spotting area. Techniques of chromatography are discussed by Rao (119), Smith (129), Giri (60), Cassidy (29), Umbreit (153), Block (19), and others.

The principle of paper chromatography consists of separating the amino acids by their relative rates of migration in a solvent system traveling across the chromatographic filter paper (29). Williams (161) lists the essential features for chromatographic equipment as:

1) The solvent reservoir must contain sufficient solvent for the chromatogram to be completed without interruption.

2) The paper must be freely suspended in the chamber.

3) Large temperature changes must be avoided since the relative rates of flow of the amino acids are dependent on the temperature.

4) The developing chamber must be sealed from the external atmosphere so that the liquid and vapor phases of the solvent remain in equilibrium during the development of the chromatogram.

The solvent used for amino acid separation was 1-butanol, glacial acetic acid, and water in the volume ratio of 60:15:25. A fresh solvent was used for each experiment (129).

The purpose of the paper chromatogram was to separate the amino acids for subsequent identification by radioautography. The distance of travel of the solvent front was restricted to nine inches because 8 inch by 10 inch X-ray film was used. One edge of the X-ray film was lined up with the line of maximum travel of the solvent front which then served as the reference line in identification of the radioactive spots.

Further details on paper chromatography techniques are discussed by Cassidy (29), Smith (129), and Carlson (28). Toxicity of solvents is discussed by Sax (127,5,6).

Amino acids travel in chromatographic solvent systems at a characteristic rate dependent upon the testing conditions. The rate of travel of an amino acid is influenced by physical factors such as temperature and type and age of solvent. Because these conditions vary with each test, a control chromatogram of known amino acids must be run with each chromatogram of unknown amino acids. Since the objective is simply to determine the fate of a single tagged amino acid, the control is simply the supernatant from sludge reduced to a pH of 2 with the tagged amino acid added. The solutions were comparable but no opportunity was given to the microorganisms and the sludge solids to react with the tagged amino acid.

APPENDIX C

C-1 SHARP'S LOWBETA COUNTER (8)

The Sharp's Lowbeta Counter has two thin windows (800 μg per cm^2) gas-flow detectors which are incorporated in a single, heavy shield to essentially eliminate background from the environmental radiation. Each gas-flow detector is operated in parallel with a cosmic ray guard counter. A cosmic ray passing through both detectors will trigger each at essentially the same time, and the electronic circuit allows this count to be registered only in the guard circuit. This anticoincidence circuit deletes almost all background caused by cosmic rays. The guard detector, placed above the gas-flow detector, is triggered each time a cosmic ray causes a count in the gas-flow detector; and the anticoincidence arrangement blanks this count from the detector scale.

The counting chambers are shielded with a minimum of four inches of lead lined with one inch of oxygen-free, high-conductivity (OFHC) copper. The OFHC copper is used to screen out gamma radiation from the contaminants in the lead.

Resolving time of the counting circuits is electronically set at 300 micro-seconds. This limits the maximum counting rate to approximately 10^5counts per minute. The sensitivity of the counter is greater than 99 percent for alpha or beta particles passing the window.

The counting efficiency will be dependent upon the absorption and energy distribution of the isotopes. The average background recorded during this experiment was 1.2 cpm for channel A and 12 cpm for channel B (contaminated).

The end-window detectors were operated in the gieger range since discrimination between alpha and beta emitters was not part of this experiment.

Q-gas composed of 98.7 percent helium and 1.3 percent butane was used as the flushing gas in the counter.

In addition to a manual sample changer for one detector, the Sharp's Lowbeta Counter has attached to it another detector system composed of an interrogator, printer, automatic sample changer with sample number counter, timer, and scaler. This system makes possible the automatic changing, counting, and recording of as many as 80 samples, or the continuous recounting of samples for the evaluation of a decay curve.

Overall efficiency for carbon-14 samples on filter paper was about five percent.

C-2 BECKMAN MODEL B SPECTROPHOTOMETER (3)

The Beckman Model B Spectrophotometer measures both transmittance and absorbance (optical density) directly on liquid samples contained in rectangular cells with a 10 mm light path.

The instrument accommodates four cells used in a standard cell holder. Any one of the four absorption cells can be precisely located in the light path without opening the cell compartment or replacing the cells. A matched set of four silica cells fitted with ground glass stoppers was used in this investigation.

A Fery prism of borosilicate glass is used as the dispersive element. The prism automatically focuses the imaged light. The wavelength range is 320 to 1000 millimicrons. A blue-sensitive phototube is used over the range of 320 to 625 millimicrons and a red-sensitive phototube is used from 625 to 1000 millimicrons. Adjustable slits allow effective handwidths of five millimicrons or less over the spectral range.

C-3 BECKMAN GC-2A GAS CHROMATOGRAPH (4)

The following description of the principles of operation is taken from the Beckman Instruction Manual for the GC-2A Gas Chromatograph.

> Sample components, introduced into the flow system of the GC-2A through the syringe inlet or gas sampling valve, are swept by the carrier gas into the chromatographic column. Here, the sample components are retarded by adsorption or absorption by the column filling material or by solution, and as the carrier gas continually flows through the column, the individual sample components emerge (elute) from the column at different times. This elution time sequence is directly proportional to the affinities of the sample components for the column packing. Therefore, under proper conditions, the stream emerging from the column is composed of carrier gas and dilute bands of components.
>
> From the column, the gas stream flows through the sensing side of the thermal conductivity detector and is exhausted at the rear of the instrument. A corresponding stream of pure carrier gas flows through the reference side of the thermal conductivity detector and out an exhaust line. Since the reference gas passes through the thermal compartment of the instrument, it is the same temperature as the sample gas stream when it reaches the detector.
>
> The elements in the thermal conductivity detector, which are arranged in a Wheatstone bridge configuration, measure the difference in thermal conductivity between the sample-carrier gas mixture and the reference stream of pure carrier gas. The bridge is balanced when pure carrier gas is in both sides of the detector. As sample-carrying gas enters the sample-measuring side of the detector, it alters the transfer of heat from a heated filament to the detector-cell wall. This change in the temperature of the filament varies its resistance and creates a voltage imbalance between the reference side and the sample side of the detector. This imbalance is recorded as a function of time on a standard strip-chart recorder. As the dilute plug of sample in carrier gas leaves the detector, the resistance of the filament returns to its original value. The area of the resulting recorder peak provides a quantitative index of the sample component. The interval between the sample injection and the elution of a sample component (retention time) can be interpreted to identify the component.

The gas chromatograph was equipped with a molecular sieve column and a silica gel column. The molecular sieve column separated oxygen, nitrogen, and methane chronologically, and absorbed water and carbon dioxide at a column and detector temperature of 70°C. The silica gel column separated air (e.g., all components of air emerged as one peak), methane, and carbon dioxide, respectively, at an operating temperature of 70°C.

Further explanation of the quantitative use of the gas chromatograph is found in Johns (76).

C-4 NUCLEAR-CHICAGO MODEL 6000 DYNACON ELECTROMETER (7)

The following description of the Model 6000 Dynacon is taken from the Nuclear Chicago Instruction Book for this instrument.

> The Model 6000 Dynacon* is a dynamic condenser electrometer designed for precise measurement of small ion currents originating in ionization chambers. Although designed specifically for radioactivity measurements, the Dynacon finds application in other areas in which sensitive measurements of voltage or current is required, e.g., mass-spectrometry, pH measurements, and studies of piezoelectic effects.
>
> Because ion chambers are unusually versatile detectors for radioactivity measurements, and because the dynamic condenser provides extreme sensitivity and stability, the system may be adapted to provide a wide variety of radioactivity measurements.
>
> ### GENERAL DISCUSSION OF ION CURRENT MEASUREMENTS
>
> An ion chamber detector is essentially a gas-filled chamber (with conducting walls) into which is inserted an insulated center rod which serves as a collecting electrode. Voltage from an external source is applied between the center electrode and the chamber wall. If the gas is subjected to ionizing radiation, either by exposure of the chamber to an external source of penetrating radiation, or by insertion of a gas, liquid, or solid radioactive sample into the chamber, gaseous ions will be produced, which migrate in the electrostatic field. The resulting ion current is proportional to the ionizing power of the radioactivity. For a given kind and energy of radiation, the ion current is directly proportional to the amount of radioactivity.
>
> The ion current may be measured by the Dynacon in either of two ways. If the current is allowed to flow through a calibrated resistor, measurement of the equilibrium voltage across the resistor permits direct calculation of the current

---

*Dynacon is a trademark of Nuclear-Chicago Corporation.

(in amperes) from Ohm's Law:

$$I \text{ (amperes)} = \frac{E \text{ (volts)}}{R \text{ (ohms)}}$$

The current is then multiplied by the appropriate calibration constant of the ion chamber to obtain the sample activity in microcuries. Alternatively, if the ion current is allowed to charge the calibrated capacitance of the Dynacon, measurement of the rate of change of the potential across the known capacity permits calculation of the current (in amperes) from the Law of Condensers:

$$Q \text{ (coulombs)} = C \text{ (farads)} \times E \text{ (volts)}$$

$$Q = I \text{ (amperes)} \times t \text{ (seconds)}$$

Therefore,

$$I \text{ (amperes)} = \frac{C \text{ (farads)} \times E \text{ (volts)}}{t \text{ (seconds)}}$$

I is then multiplied by the appropriate constant to obtain the sample activity. In general, equilibrium voltage (steady-deflection) measurements are used for determination of large ion currents, and the rate-of-change method is used when highest sensitivity and statistical accuracy are required.

Ion chambers of 100-, 250-, 500-, and 1000-milliliter volumes are available for measurement of radioactive gases in essentially 4π geometry. Solid or liquid samples up to 70 cm^2 in area may be measured in 2π geometry in the Model DCL slide chamber. Flow chambers, useful for the study of dynamic gas systems, are available in four sizes from 100 to 1000 milliliters.

In operation the equation is as follows:

$$\text{I (amperes)} = \frac{\text{(voltage difference in volts)(input capacitance in farads)}}{\text{time difference in seconds}}$$

Example:

Voltage difference = 1000 millivolts = 1.0 volts

Time difference = 500 seconds

Input capacitance = $22.2\,(10^{-12})$ farads = 22.2 pf

$$I = \frac{(1.0\ \text{volts})[22.2\,(10^{-12})\ \text{farads}]}{500\ \text{seconds}}$$

$$I = 4.43\,(10^{-14})\ \text{amperes}$$

For $C^{14}O_2$ gas in 100 ml ionization chamber the ion chamber constant was $3.74\,(10^{-12})$ amps/µc.

For $C^{14}H_4$ the constant was $0.941\,(10^{-12})$ amps/µc.

Therefore, if the above gas was $C^{14}O_2$, its activity was 0.01186 µc = 11.86 nc.

APPENDIX D

REFERENCE TABLES

Table D-1

Average Chemical Constituents of Sewage Solids and Sludges, Percent on Dry Weight Basis (123)

Constituent	Fresh	Activated	Digested
Organic Matter	60 - 80	65 - 75	45 - 60
Total Ash	20 - 40	25 - 38	40 - 55
Insoluble Ash	17 - 35	22 - 30	35 - 50
Pentosans	1.0	2.1	1.5
Grease and Fat (Ether)	7 - 35	5 - 12	3.5 - 17
Hemicelluloses	3.2	-	1.6
Cellulose	3.8	7.0 (Incl. lignin)	0.6
Lignin	5.8	-	8.4
Protein	22 - 28	37.5	16 - 21
Nitrogen (N)	4.50	6.20	2.25
Phosphoric (P_2O_5)	2.25	2.50	1.50
Iron (Fe_2O_3)	3.20	7.20	6.00
Chlorides (Cl)	0.50	0.50	0.50

Table D-2

Approximate Organic Analysis of Fresh Solids and Ripe Sludge, Percent on Dry Weight Basis (70)

Constituent		Fresh Solids	Ripe Solids
Ether Soluble		34.40	8.18
Soluble in Cold and Hot Water		9.52	5.48
Alcohol Soluble		2.49	1.59
Hemicellulose		3.20	1.58
Cellulose		3.78	0.56
Lignin		5.78	8.37
Crude Protein		27.12	19.68
Ash		24.13	56.00
	Total	110.42	101.44

Table D-3

Analysis of Fresh Sludge and Ripe Sludge, Percent on Dry Weight Basis (22)

Constituent	Fresh Solids	Ripe Solids
Protein (N x 6.25)	19.4	12.5
Grease	25.2	26.9
Crude Fiber	10.8	9.8
Humic Acids	4.0	8.6
Total	59.4	57.4
Volatile Matter by Analysis	60.9	59.5

Table D-4

Organic Constituents of the Settleable Fraction of Sewage, Percent on Dry Weight Basis (11)

	Ether Soluble	
Total Grease	--	19.13
Free Fatty Acids	--	--
Saturated	0.48	--
Unsaturated	0.10	--
Glyceride Fatty Acids	--	--
Saturated	9.95	--
Unsaturated	3.12	--
Phospholipids	0	--
Unsaponifiable	2.78	--
Total	16.43	19.13

Recovery 86 percent

	Alcohol Soluble	Alcohol Insoluble
Total Nitrogenous Matter (Total N x 6.25)	2.93	22.15
Sum of Amino Acids	1.97	17.34
Total N due to Amino N	68.3	78.5

	Carbohydrates
Pectin	3.10
Hemicellulose	14.20
Lignin	6.10

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